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Purification of Hypothalamic Corticotropin Releasing Factor.* (25553)

PAUL C. ROYCE† AND GEORGE SAYERS
Dept. of Physiology, Western Reserve University School of Medicine, Cleveland, O.

Appropriate stimuli promptly increase rate of release of corticotropin from the adenohypophysis(1). The response is abolished by destruction of an area at the base of the hypothalamus which includes median eminence. The defect in an animal with such a lesion has been ascribed to loss of a chemical mediator, corticotropin releasing factor (CRF), which originates in the median eminence and is transported in the hypophyseal portal venous system to the adenohypophysis where it acts to accelerate release of corticotropin(2). The presence of corticotropin releasing activity in crude extracts of median eminence tissue lends support to this hypothesis(3). The present communication describes column chromatography of crude extracts which led to the preparation of fractions exhibiting neither pressor nor corticotropic activity at a dose which induces substantial adrenal ascorbic acid depletion in the median eminence lesioned rat.

Methods. Calf brains were dissected at the slaughterhouse.‡ The pituitary stalk was transected immediately dorsal to the diaphragm sella. A portion of tissue including both median eminence and pituitary stalk (SME) was separated from the brain and dropped into acetone (100 to 150 SMEs/2 liters of acetone). The acetone was decanted and replaced daily for 3 days. The dehydrated, defatted tissue was air dried and stored in tightly covered containers at 4°C. Crude extracts were prepared by refluxing the acetone dried tissue for 10 to 15 minutes in glacial acetic acid at 100°C. One ml of glacial acetic acid was used for each SME. The mixture was filtered and the filtrate (crude extract) stored at room temperature in glass stoppered flasks. CRF activity of crude extracts was undiminished after 3 months of storage. As a prelude to chromatography, 1 volume of acetone and 2 volumes of ether were added to the crude extract. One to 12 hours later the precipitate was separated by low speed centrifugation and washed 4 times with a volume of ether equal to original volume of crude extract. The ether washed, acetone-ether precipitate was extracted with 0.01 M ammonium acetate buffer (pH 5.8) (see below for volume). The buffer extract centrifuged at 10,000 x gravity for 10 minutes, and the clear supernatant applied to a carboxymethylcellulose (CMC) column. CMC was prepared by the method of Peterson and Sober(4) as modified by Ellis and Simpson (5). It was equilibrated with 0.01 M ammonium acetate buffer (pH 5.8) and the fines removed by repeated decantation. The CMC was loaded into a 0.9 x 30 cm chromatography tube to a height of 10 cm and washed extensively with 0.01 M ammonium acetate buffer (pH 5.8). In Exp. W-3, 100 ml of crude extract, prepared from 100 SMEs, were processed for chromatography. Ether washed precipitate was extracted 4 times with 1 ml of buffer and 3 ml of buffer extract were applied to the column. In Exp. W-4, 60 ml of crude extract, prepared from 60 SMEs, were processed for chromatography. The ether washed precipitate was extracted 4 times with 3 ml of buffer and 9 ml of buffer extract were applied to the column. In both experiments, effluents were collected at 8 to 10 ml/hour. When the level of buffer extract reached the top surface of CMC, 10 ml of 0.01 M buffer were added. The effluent from this buffer wash was combined with the effluent from the buffer extract. Discontinuous gradient elution was then begun. The column was washed successively with 15 ml each of 0.05, 0.1, 0.2, and 0.4 M ammonium acetate buffer and the effluent of each successive buffer was collected separately. pH of all buffers was 5.8. As a precaution against deterioration of CRF, collection flasks contained sufficient glacial acetic acid so that final concentration of acetic acid in each was 3%. Acetone-ether precipitation and subsequent steps were carried out at 4°C. Assays for CRF activity were performed in median eminence lesioned rats (250 to 300 g) (2). Rats with urine volumes greater than 75 ml in the first 24 hours after lesioning were used for assay. Forty-eight hours post-operatively the left adrenal was removed and material to be assayed infused intravenously over 30 seconds. Thirty minutes later the right adrenal was removed. The difference between concentrations of adrenal ascorbic acid in left and right adrenals is an index of corticotropic action during period between removal of the 2 glands. The response may represent corticotropin release and/or corticotropin injected. Corticotropic activity of extracts was determined by assay in 24-hour hypophysectomized rats (110 to 135 g)(6). Vasopressin content was estimated by pressor response of rats pretreated with phenoxybenzamine (U.S.P. XV method). The dose of each fraction used in the 3 assay systems was equivalent to 0.3 SME.

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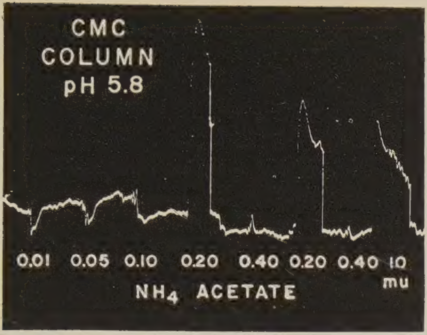
† Upjohn Postdoctoral Fellow 1958-1959.

‡ Drs. C. Graham and E. A. Lazo-Wasem (Wilson Labs., Chicago) arranged for and supervised tissue collections.

Results. Fig. 1 illustrates blood pressure response to the fractions of Exp. W-4. No detectable pressor activity was found in material emerging with 0.01, 0.05 or 0.1 M buffer. The bulk of pressor activity emerged with the 0.2 M buffer but a small pressor response was elicited by the 0.4 M fraction. Blood pressure response to 10 milliunits Pitressin (Parke, Davis) is included for comparative purposes. Distribution of pressor activity was the same in Exp. W-3.

In both experiments W-3 (Fig. 2) and W-4 (Fig. 3), corticotropic activity as measured in the hypophysectomized rat was found exclusively in the 0.4 M fraction. Because of high corticotropic activity of the buffer extract and of the 0.4 M fraction, they were not assayed for CRF activity in the lesioned rat. The 0.1 M fraction which had no detectable pressor or corticotropic activity at a dose of 0.3 SME displayed CRF activity at this dose in the lesioned rat. The protein concentration of the 0.1 M fraction was 15 μg/SME for W-3 and 13 μg/SME for W-4. In both experiments the 0.05 M fraction was inactive in the lesioned rat, but significant CRF activity was found in 0.2 and 0.01 M fractions. The presence of CRF activity in the 0.2 M fraction probably results in part from the vasopressin in this fraction and in part from an



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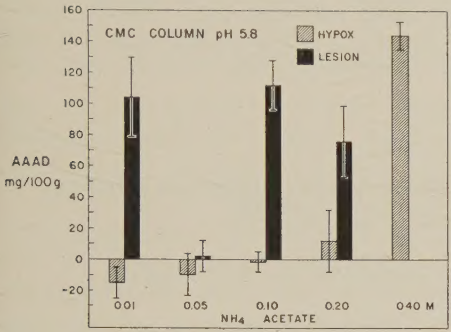

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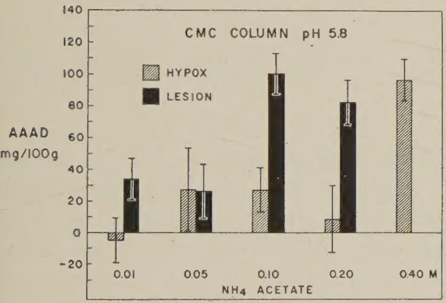

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FIG. 1. Kymograph record of blood pressure response of rat after administration of fractions of median eminence (SME) extract W-4 eluted from carboxymethylcellulose column with ammonium acetate buffers of increasing molarity. Dose of each fraction equivalent to 0.3 SME. Response to 10 milliunits of Pitressin is shown at extreme right.

FIG. 2. Adrenal ascorbic acid depletion in median eminence lesioned and in hypophysectomized rats after administration of various fractions of hypothalamic (SME) extract W-3. Fractions are designated by molarity of ammonium acetate buffer with which they were eluted from carboxymethylcellulose column. Height of bar indicates avg response of 6 animals to 0.3 SME. Stand. error shown by vertical lines.

FIG. 3. Responses to extract W-4. Same conditions as for W-3 (Fig. 2) except height of bar indicates avg response to 5 or more animals.

overlap of CRF of the 0.1 M fraction into the 0.2 M fraction.

One explanation for presence of corticotropin releasing activity in the 0.01 M and in the 0.10 M fractions is the existence of 2 chemically distinct CRFs in SME tissue. An alternate explanation is that the fractionation technic is imperfect and the CRF in the 0.01 M fraction is identical with that in the 0.1 M fraction. The much greater CRF activity of the 0.01 M fraction of Exp. W-3 as compared to that of the 0.01 M fraction of Exp. W-4 may be related to differences in concentration of the ether washed precipitate in the buffer extract (W-3, 25 SMEs/ml; W-4, 5 SMEs/ml). No signs of toxicity were observed in the lesioned rat following rapid infusion (30 seconds) of 0.3 SME of the 0.1 M fraction.

Discussion. Our previous report indicated that calf SME tissue contains at least 3 components capable of activating the adrenals of the median eminence lesioned rat(3). One appeared to be corticotropin, another, vasopressin. Presence of an additional active factor was inferred from the following evidence. (1) At appropriate dilutions, activity of crude SME extracts in the lesioned rat was greater than could be accounted for by their corticotropic and/or by their pressor activities. (2) Pepsin treatment of crude SME extracts reduced adrenal stimulating activity in the lesioned rat but did not diminish corticotropic or pressor activity. It was postulated that the pepsin-labile component acted directly on the adenohypophysis to accelerate the release of corticotropin. Biological characterization of this CRF was limited by presence of corticotropin and vasopressin, as well as by its toxicity. The corticotropin activity most likely is due to contamination with adenohypophyseal tissue since it is possible with careful dissection to obtain material high in corticotropin releasing activity but with negligible corticotropic activity. Preparation of CRF by the method described in this report provides non-toxic material which contains non-detectable quantities of pressor and corticotropic activities and which is suitable for further biological investigation. Elucidation of the relationship between the CRF of SME origin and that reported in portal vein blood(7,8) and in neurohypophyseal tissue(9) must await isolation of active material in homogeneous form from each of these three sources.

Summary. Fractionation of a crude extract of calf median eminence on carboxymethylcellulose columns yields a preparation which induces adrenal ascorbic acid depletion in the median eminence lesioned rat. This partially purified corticotropin releasing factor (CRF) is substantially free from corticotropic and pressor activities.

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Evaluation of Type and Degree of Change in Postexercise Electrocardiogram in Detecting Coronary Artery Disease. (25554)

GEORGE P. ROBB AND HERBERT H. MARKS (Introduced by E. F. Beach)
Metropolitan Life Insurance Co., N. Y. City

The value of electrocardiographic double standard 2-step exercise test(1) in detection of occult coronary disease has been proved (2,3,4). The criteria for abnormal response to this exercise test indicative of coronary insufficiency, however, have not been adequately tested or defined. Investigation of validity of the original criteria of a positive or abnormal test by a clinical-statistical follow-up study was started in 1952 and completed in 1956 (4). Incidence of coronary occlusion and coronary death subsequent to the test were determined in a large series of military personnel and insurance applicants suspected of having coronary disease who had been given the double exercise test. The results indicated that the only valid evidence of coronary insufficiency in postexercise electrocardiogram was ischemic ST segment depression. The significance of ST junction depression remained undetermined because, through technical fault in the series on military personnel, unrecognized ST segment depression cases were included in ST junction category. Isolated T-wave change after exercise appeared normal. The present report gives new data regarding significance of (1) various types of ST junction depression after exercise; (2) various types and degrees of ischemic ST segment depression; (3) additional data regarding negative reactors and (4) QS/QT ratio in various types of response to the test(5).

Materials and methods. This is a follow-up study of 922 insurance applicants, most of whom were suspected of having coronary disease and who were given the double standard 2-step exercise test between 1949 and March 1958, and traced to March 1959. Indications for the test, its performance, and electro-cardiographic technic, were described previously(4). The post-exercise electro-cardiograms were analyzed with the P-R segment as the point of reference for measuring ST junction and ST segment depression(1). The term "ischemic" denotes horizontal or sagging depression of the ST segment(6). The changes induced by exercise were classified as in Chart 1: 1. *ST junction depression, any degree.* Types: (a) transitory, during tachycardia; (b) prolonged; (c) "near-ischemic" configuration; *i.e.,* ST segment straight and nearly horizon-

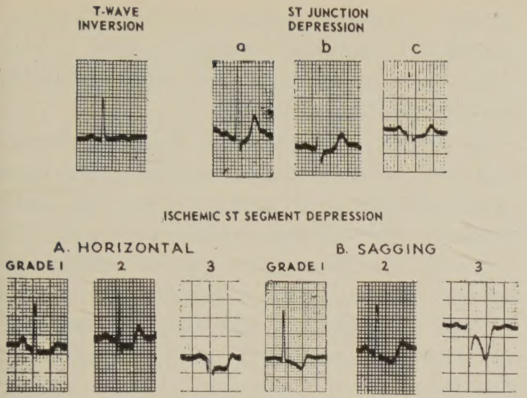

FIG. 1. Types of electrocardiographic response to double two-step exercise test.

tal. 2. *ST segment depression.* Type A: Horizontal segment – without significant T-wave change. Grades: 1– 0.0 to 0.9 mm; 2– 1.0 to 1.9 mm; 3– 2.0 mm or more. Type B: Sagging segment – with diphasic or inverted T-wave. Grades 1, 2, 3, as indicated under "Horizontal segment." Data on the 922 cases were analyzed according to standard actuarial procedure to obtain mortality rate/1,000 person years of observation from all causes and from coronary disease for each type and degree of response. This statistical basis assures comparability of results in the several classifications.

Results are summarized in Table I. Altogether, there were 29 deaths to date of follow-up. Of these, 13 occurred in the ischemic ST segment depression cases, 12 of them from coronary disease. Only 2 deaths, both from coronary disease, occurred in the more numerous ST junction group; 2 in the T-wave group (one of them from coronary disease); and 12 in the negative reactors (6 from coronary disease).

Among the 226 ST junction cases of all types the death rate from coronary disease was only 2.8 per 1,000 years of observation, essentially the same as the rate of 3.1 in the negative group. Analysis of transitory, prolonged, and "near-ischemic" subgroups showed no significant differences, but the experiences are all small in size. Among the 145 cases with ischemic ST segment depression, death rate from coronary disease was 23.8 or about 8 times the rate for the ST junction and negative series. The experience on horizontal and sagging types of ST depression have been combined to obtain a greater volume of data for statistical analysis according to degree of depression. This analysis showed a progressive and accelerating increase in coronary death rate with increasing ST segment depression. Thus, among the 85 cases of Grade 1 the death rate was 12.9; among the 42 cases of Grade 2, 21.2 or about one-half more than for Grade 1; while for the 18 cases of Grade 3, the coronary death rate was 95.1 or more than 7 times the rate for Grade 1. The T-wave change group was too small for statistical analysis.

The QS/QT ratio showed distinct differences between negative reactors and those with ST segment depression, particularly of the sagging type, but not between negative reactors and those with ST junction depression. Moreover, the ratio does not discriminate well between cases with various degrees of ST

TABLE I. Death Rates/1,000 Person Years of Observation among Insurance Applicants Given Double Standard 2-Step Exercise Test. Negative reactors and those with ST depression, classified by type and degree of change in postexercise ECG. Metropolitan Life Ins. Co., cases tested in 1949-March 1958, traced to March 1959.

Classification	No. of cases	Deaths		Death rates	
		All causes	Coronary	All causes	Coronary
Negative	519	12	6	6.3	3.1
ST Junction Depression (more than 0.5 mm)	226	2	2	2.8	2.8
a. Transitory, during tachycardia	114	1	1	3.0	3.0
b. Prolonged	100	1	1	2.9	2.9
c. ''Near-ischemic'' configuration	12	0	0		
Ischemic ST Segment Depression	145	13	12	25.7	23.8
1. .0 to .9 mm	85	4	4	12.9	12.9
2. 1.0 to 1.9 mm	42	3	3	21.2	21.2
3. 2.0 mm or more	18	6	5	114.1	95.1

segment depression. In isolated T-wave inversion the method is not applicable.

Discussion. This follow-up of 226 cases of ST junction depression provides additional evidence that ST junction depression is a normal response to exercise. Even the prolonged and "near ischemic" types ascribed by some investigators to mild coronary insufficiency had a low death rate. In contrast, all grades of ischemic ST segment depression had a high coronary death rate. This type of electrocardiographic response to exercise evidently is due to coronary insufficiency caused by coronary atherosclerosis. The increase in coronary death rate that accompanies the increase in ST segment depression suggests that degree of ischemic depression is directly related to severity of coronary insufficiency induced by the test. It is of interest that the 8 coronary deaths in the Grade 2 and 3 cases showed sagging ST segments and diphasic or inverted T-waves. This type of reaction probably denotes a more severe degree of coronary insufficiency than does the horizontal type. Further analysis according to type and degree will be made when enough material is available. While QS/QT ratios differ for the extremes—negative reactors and those with ischemic ST segment depression—this technic does not offer any special advantage over inspection of the tracings.

Summary. Ischemic ST segment depression after exercise is due usually to coronary atherosclerosis and insufficiency. Our data suggest that type and degree of ischemic ST segment depression after exercise reflects the severity of the coronary disease. The results of this investigation indicate also that ST junction depression is a normal response to exercise.

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Immunocytochemical Demonstration of Intranuclear Localization of 18S Gamma Macroglobulin in Macroglobulinemia of Waldenström (25555)

THOMAS F. DUTCHER AND JOHN L. FAHEY
Pathologic Anatomy, and Metabolism Service, Natl. Cancer Inst., Bethesda, Md.*

We recently reported the presence of intranuclear material within lymphoid plasma cells of 3 patients with macroglobulinemia of Waldenström(1). This material was histochemically identified as a protein with high carbohydrate content. The carbohydrate component was shown not to consist of glycogen since it was periodic acid-Schiff(PAS) positive after digestion with diastase. The isolated serum 18S gamma macroglobulin from each of the 3 patients also had a high carbohydrate content and was strongly PAS positive following electrophoresis on filter paper strips.

The present study, utilizing the fluorescent antibody technic of Coons and Kaplan(2), supports the probable identity of protein material within the nucleus and cytoplasm of lymphoid plasma cells and the circulating serum 18S gamma macroglobulin from a patient with macroglobulinemia of Waldenström.

Methods. Antisera preparation: Purified gamma macroglobulin from serum of patient J.W. was obtained by repeated precipitations of the protein from solution by dialysis against low ionic strength pH 8.0 buffers. The final product was homogeneous by analytic electrophoresis and upon ultracentrifugation consisted of 18S macroglobulin (with small amounts of 23S and 27S components). Whole normal human gamma globulin, prepared by electrophoretic procedures contained 90% 6.6S gamma globulin and 10% 18S gamma macroglobulin(3). Antisera to purified J.W. macroglobulin and to whole normal human gamma globulin were produced utilizing technics described elsewhere(3). Rabbit anti-macroglobulin serum was absorbed with commercially prepared gamma globulin (Cohn Fraction II) to remove antibodies to 6.6S gamma globulins. This absorbed anti-macroglobulin serum produced single precipitin bands in Ouchterlony gel diffusion plates(4) when tested against purified J.W. macroglobulin and against whole normal gamma globulins. These bands showed a reaction of identity in the gel diffusion plate demonstrating specificity of the absorbed rabbit anti-macroglobulin for the J.W. macroglobulin and for at least some of the 18S gamma macroglobulin present in whole normal gamma globulin. Rabbit anti-whole normal gamma globulin serum contained antibodies against both 6.6S and 18S gamma globulins. This antiserum was absorbed with purified J.W. macroglobulin to removed antibodies which might have reacted with J.W. macroglobulin. The antibodies in the 2 absorbed antisera were precipitated and concentrated by ⅓ saturation with $(NH_4)_2SO_4$ redissolved and dialyzed against saline. We are extremely grateful to Dr. Arthur Silverstein, Chief, Immunochemical Section, Armed Forces Inst. of Pathology, for performing the fluorescein labeling of the concentrated antisera. The labeled antisera were absorbed with acetone dried human liver powder. *Bone Marrow Smears*: At the time of our original report, J.W. did not have demonstrable intranuclear PAS positive material in cells aspirated from bone marrow but this has subsequently been observed in repeated marrow aspirates. Aspirated marrow particles were smeared on coverslips, air dried and stored at 4°C until stained. The coverslips were placed in 4°C acetone for 10 minutes, rapidly air dried and covered with absorbed fluorescein labeled antisera for 45-60 minutes in a moist chamber at room temperature. They were then agitated gently during two 10 minute rinses in pH 7.4 phosphate buffered normal saline, mounted on slides with 25% glycerol in buffered saline and observed with Leitz ultraviolet microscope using wavelength of 390-440 mμ. Several cells with intranuclear fluorescence were photographed and the microscopic fields were marked. The coverslips were removed from the slide by immersion in buffered saline, air dried, fixed for 3-5 minutes in formalin vapor, washed in running water and stained by a PAS-Hematoxylin technic. The marked fields were then examined with standard illumination. Controls for specificity of fluorescence consisted of bone marrow smears from J.W. incubated with the absorbed fluorescein labeled anti-normal gamma globulin and smears incubated with pooled rabbit sera containing unlabeled anti-macroglobulin for 1 hour prior to incubation with the fluorescein labeled anti-macroglobulin. Smears of aspirated bone marrow particles from patients with multiple myeloma and carcinoma served as non-macroglobulinemic controls.

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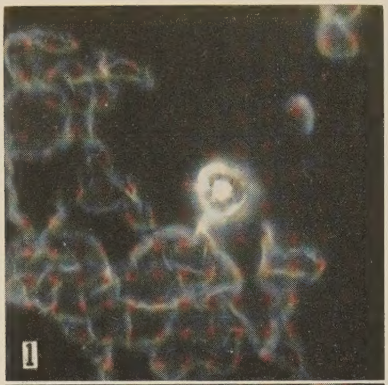
* Nat. Inst. H., U. S. Dept. H.E.W.

Results. Specific, brilliant green fluorescence was present within the otherwise non-fluorescing nucleus of several cells in smears incubated with the fluorescein labeled anti-macroglobulin preparation (Fig. 1). Diffuse specific fluorescence was also present in cytoplasm of most cells with intranuclear fluorescence and in cytoplasm of a few cells with an eccentric, non-fluorescent nucleus. Preincubation with unlabeled anti-macroglobulin serum diminished intensity of the specific fluorescence. The fluorescent nuclear area was usually demonstrated to be PAS positive.

Some cells with the intranuclear area of fluorescence were noted to have a zone of perinuclear fluorescence which was not sharply circumscribed (Fig. 2). In PAS stained preparation, these cells were distorted and consisted of an apparently intact nucleus with a surrounding clear zone, within which there were clumps of PAS positive material but no recognizable cytoplasmic structures. Most cells of this type contained intranuclear PAS positive material but in a few the area previously occupied by specific fluorescence was colorless. It is assumed that the macroglobulin was in this intranuclear area at the time of the reaction between it and the labeled



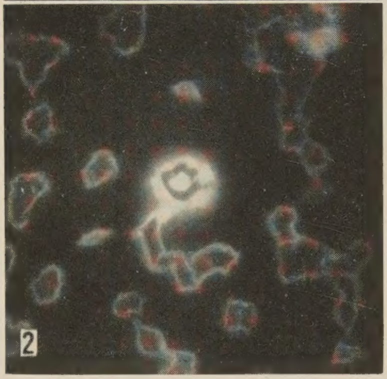


FIG. 1. Fluorescence photomicrograph of bone marrow smear incubated with fluorescein labeled anti-18S macroglobulin. Compact fluorescence in nuclear area and surrounding diffuse, but rather well circumscribed cytoplasmic fluorescence. Note erythrocytes outlined by fluorescence. Enlarged 3× from ×500.

FIG. 2. Same as Fig. 1. Less well circumscribed perinuclear fluorescence of a distorted lymphoid plasma cell.

anti-macroglobulin, but was subsequently removed during the wash in buffered saline or during PAS procedure. PAS positive clumps in the clear perinuclear area of these distorted cells may represent the material which was originally within the nucleus.

The reason for the observed cellular distortion is not known but the phenomenon has been reported by Louis(5) in peripheral blood smears stained by the Leishman technic following ultraviolet microscopy. Several neutrophils were similarly distorted and it may be, as suggested by Louis, that ultraviolet irradiation is detrimental to cellular structures even after "fixation."

Smears incubated with labeled absorbed anti-human gamma globulin contained cells with an eccentric nucleus and a diffuse cytoplasmic fluorescence similar to that described for plasma cells(6) and myeloma cells(7). The J.W. marrow and carcinoma control marrows contained relatively few of these cells whereas they were numerous in multiple myeloma control smears. No intranuclear fluorescence was seen in anti-human gamma globulin preparations.

Other cellular components of marrow smears had no specific fluorescence with either labeled antiserum. Neutrophilic granules had a faint non-specific fluorescence and other granulocytes, either eosinophils or basophils, contained bright yellow to orange cytoplasmic granules. The background plasma was PAS positive, intensity depending upon thickness of smear. This was consistent with the presence of 8 g % of gamma macroglobulin of high carbohydrate content in the patient's plasma. Plasma fluorescence was concentrated at the red cell surface as noted by Pachter *et al.*(8).

Discussion. The demonstration of immunologic, in addition to histochemical, identity of intranuclear PAS positive material of lymphoid plasma cells and the circulating macroglobulin in a patient with the macroglobulinemia of Waldenström is strong support for the concept that the lymphoid plasma cell contains macroglobulin. Whether the intranuclear material represents macroglobulin formation, storage or destruction and whether this is a normal or abnormal site of macroglobulin metabolism, awaits further study. In view of the analogous situation, wherein myeloma protein has been shown in myeloma cells by the immunofluorescent technic(7) and formed in myeloma tumor by biochemical technics(9), it is reasonable to expect that macroglobulin is formed in lymphoid plasma cells.

At the time of this study, the marrow from patient J.W. contained fewer lymphoid plasma cells than we observed in other patients with macroglobulinemia. Each J.W. bone marrow smear incubated with anti-macroglobulin preparation contained 20 to 30 fluorescent cells and most of these had intranuclear fluorescence. A similar number of fluorescent cells were found in parallel smears incubated with antinormal 6.6 S globulin.

Specific intranuclear fluorescence with the anti-J.W. macroglobulin was demonstrable in cells of one other patient with macroglobulinemia. Recently Curtain(10), using a similar technic, observed cytoplasmic, but not intranuclear, fluorescence of plasmacytoid cells from bone marrow of 2 patients. One of these patients had increased serum gamma macroglobulins but an atypical clinical history for Waldenström's macroglobulinemia(11). The other patient appeared to have multiple myeloma.

Summary. Immunofluorescent and cytochemical technics were used to identify 18S gamma macroglobulin within the nucleus and cytoplasm of lymphocytoid plasma cells from a patient with macroglobulinemia of Waldenström. Intranuclear fluorescence coincided with presence of intranuclear PAS positive protein. This observation supports the concept, suggested by histochemical studies, that circulating macroglobulin and intranuclear protein of lymphocytoid plasma cells are closely related and may be identical. The evidence suggests that macroglobulin is formed in lymphocytoid plasma cells.

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Response of Isolated Auricles to Some Substances of Metabolic Interest.* (25556)

V. M. VENTURI† AND HOLLIS G. SCHOEPKE (Introduced by F. E. Shideman)
Dept. of Pharmacology and Toxicology, University of Wisconsin, Madison

Inotropic actions of various intermediary metabolites have been studied most often using preparations of rat ventricular muscle (1,2,3), papillary muscle of cat(4) or isolated heart(5,6,7). Similar studies on isolated auricles of rabbit(8) have been conducted in a somewhat different manner and differences in activity noted. Ventricles and auricles also differ with respect to their sensitivity to various inorganic ions(9,10) and exhibit a difference in responsiveness to stimulation of their extrinsic nerve supply(11). These observations led us to perform the following experiments to determine whether or not the auricular effects in the rat of malonic acid and certain intermediates of the tricarboxylic acid cycle are similar to those elicited by these agents in ventricular preparations from the same animal. We also attempted to explain the observed effects utilizing a somewhat different experimental approach than that employed previously.

Materials and methods. Albino rats of Holtzman strain, unselected as to sex and weighing approximately 150 g were used. Animals were anesthetized with ether and the hearts rapidly removed. After excising ventricular muscle, blood vessels, fat and connective tissue, the auricles were suspended in a muscle chamber containing 250 ml of Locke's solution maintained at constant temperature and aerated with mixture of 95% oxygen and 5% CO_2 by means of sintered glass plate at bottom of chamber. The Locke's solution had the following composition: NaCl, 0.9%; KCl, 0.042%; $CaCl_2$, 0.024%; $NaHCO_3$, 0.05%. In those experiments employing a glucose-containing medium, the concentration of this substance was 0.10% unless otherwise stated. Contractile amplitude and rate of spontaneous beat were recorded with isotonic lever on a kymograph. Auricles were allowed to equilibrate at least one hour after placing in the medium prior to addition of any of the compounds and were washed frequently after each response. Compounds were employed as obtained from commercial sources, dissolved in medium used in muscle bath, adjusted to pH 7.0, and added to bath in a volume not exceeding 2 ml.

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† Present address: Dept. of Pharmacology, University of Pavia, Italy.

Results. The isotonic contractile amplitude of auricles of the rat was reduced by various intermediary metabolites and malonate (Table I). The effect produced by acetate was more transient than that seen with other compounds. Although addition of glucose (5 or 10 μmol/ml) to the medium increased contractile amplitude at 28°C, it caused little or no change in contractility of auricles studied at 38°C. Inhibitory activity was greatest with citrate and least with lactate. In general a negative inotropic response was accompanied by decrease in rate, though of less magnitude, and the effects produced by the compounds in a medium maintained at 28°C were very much like those at 38°C.

TABLE I. Negative Inotropic Effects of Various Compounds on Isolated Rat Auricles.

	Conc. of compound (μmol/ml)			
	1	5	10	20
Compound*	% decrease in contractile amplitude			
Citrate	69 ± 2.1†	100 ± 0	100 ± 0	100 ± 0
Malonate	7 ± 1.0	22 ± 4.0	44 ± 3.4	90 ± 6.9
Malate	15 ± 2.0	25 ± 3.9	54 ± 7.0	18 ± 2.0
Succinate	10 ± 3.0	28 ± 2.9	58 ± 5.2	73 ± 6.4
Pyruvate	4 ± 2.6	20 ± 4.3	33 ± 7.9	40 ± 5.0
Fumarate	0	15 ± 4.7	25 ± 1.3	41 ± 2.2
Acetate	0	8 ± 1.6	36 ± 5.3	45 ± 3.6
Lactate	0	6 ± 1.6	24 ± 4.2	39 ± 4.2

* All compounds were employed as sodium salts.
† Mean ± S.E.

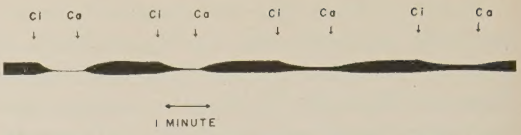

FIG. 1. Effect of repeated alternate administration of calcium and citrate on contractile amplitude of isolated auricles of the rat. Following the response to 2.5 mmol of sodium citrate (Ci), 2.25 mmol of calcium chloride (Ca) was added. Seven such titrations were performed before signs of calcium intoxication appeared.

Degree of inhibition of contractile amplitude was not significantly affected by addition of glucose to the medium. Sodium malonate produced a reduction in amplitude of contractions of the auricles. This was seen in a medium containing either 2.45 μmol/ml or 2.16 μmol/ml of calcium chloride. The effects were very similar at 28°C and 38°C and were not significantly affected by addition of glucose to the medium. Addition of sodium (as sodium chloride) equal in amount to that in the compounds under study did not alter contractile amplitude.

Negative inotropic effect of the metabolites was reversed by addition of appropriate amounts of calcium chloride to the medium. Reciprocally, enhanced contractility produced by certain quantities of calcium was antagonized by addition of metabolites under study. Using the "titration" method of Swain *et al.*, (13), experiments were performed in which calcium chloride and one of the metabolites were alternately added to the bath. One such experiment is illustrated in Fig. 1 where 2.5 mmol of sodium citrate was "titrated" in this manner with 2.25 mmol of calcium chloride. A series of 7 such "titrations" could be performed before signs of calcium toxicity were observed. Similar "titration" experiments were conducted using succinate, malonate, malate, fumarate, pyruvate and lactate. In each experiment the following were determined: (1) lowest concentration of calcium necessary in the medium to cause a just perceptible reversal of the negative inotropic effect produced by 2.5 mmol of the compound used, (2) concentration of calcium required for complete reversal of the effect produced by the compound, and (3) total calcium concentration present in the medium during a

TABLE II. Reversal of Negative Inotropic Effects of Various Compounds on Rat Auricles by Calcium Chloride.

Compound*	$CaCl_2$ conc. (μmol/ml) producing: Partial reversal	Complete reversal	Toxic effects
Citrate	3.0	9.0	65.2
Succinate	.4	.8	7.0
Malonate	.1	1.0	6.2
Malate	.2	.6	6.4
Fumarate	.2	.7	7.1
Pyruvate	.2	2.0	8.4
Lactate	.2	.8	6.2

* All compounds were employed at a final concentration of 10 μmol/ml except lactate in which case concentration was 20 μmol/ml.

"titration" experiment when toxic effects of calcium, *e.g.* arrhythmias, first appeared. These results are summarized in Table II. Calcium was least effective in counteracting the negative inotropic response to citrate. It also will be noted that citrate was capable of antagonizing approximately 10 times more calcium than were the other compounds.

Discussion. These experiments demonstrated that all compounds examined, except glucose, exert a negative inotropic effect on the isolated auricles of the rat. These results confirm those obtained by a previous investigator (8) who employed the isolated auricles of the rabbit. Similar effects have been observed on isolated perfused rat heart with a glucose-free perfusing medium(6) and on the isolated perfused rabbit heart(7). The increase in amplitude observed after addition of glucose at 28°C parallels the observations of previous investigators(4) on papillary muscle of the cat. However, in contrast to these findings, it has been reported(1) that hypodynamic rat ventricular strips show recovery of contractile amplitude upon addition of pyruvate, lactate, β-hydroxybutyrate, acetate, succinate or glucose in phosphate buffered solutions. This difference in results may be related to a difference in sensitivity of auricles and ventricles to these compounds or to differences in media or technics employed since the effect of succinate, at least, varied with different media employed in these experiments. Hydrogen ion concentration also appears important since at pH 7.6 in a bicarbonate buffered medium, no recovery of depressed amplitude occurred with citrate, succinate or malate on rat ventricular strips(3) whereas the latter 2 substrates were effective in a medium buffered at pH 6.2. Positive inotropic actions of pyruvate, glucose and acetate but not citrate, fumarate, succinate and malate have been observed with hypodynamic rabbit auricles(8).

The negative inotropic effect of malonate which we observed is in contrast to lack of activity of this compound on the driven auricular preparation reported by other investigators(12) and positive inotropic activity on rat ventricular strips seen by still other workers(2). The latter investigators employed a higher concentration of calcium (2.45 μmol/ml) than we used. However, even at this higher calcium concentration, we observed only negative inotropic responses of rat auricles to malonate. The results suggest that reduction of contractility by malonate is not the result of inhibition of metabolic activity in the tricarboxylic acid cycle, since metabolites of the cycle are capable of producing similar negative inotropic responses, as reported previously(8).

With the exception of acetate, all compounds utilized in this study which elicited negative inotropic responses are theoretically capable of forming chelate-type compounds with the calcium ion. Sodium acetate, a monodenate compound, would not be expected to chelate with calcium but complexes of a nonring-type might be formed. Our data suggest that the negative inotropic activity of the compounds examined could be due to a decrease in concentration of available calcium in the medium. This concept agrees with the work of other investigators who believe that depression of amplitude by malonate(2) and by citrate(8) is probably due to a reduction in calcium concentration of the medium. The myocardial depressant effects of compounds employed were overcome by addition of appropriate amounts of calcium chloride to the medium. Conversely the toxic effect of certain concentrations of calcium chloride could be limited by subsequent addition of one of the compounds in question. "Titration" experiments provide even more convincing support for the view that there is some interaction between calcium ion and these compounds. Reversal of only a single episode of depression with calcium would not be significant since the negative inotropic action of many substances can be antagonized nonspecifically with calcium salts but several consecutive reversals provide more definitive information(13). It was reasoned that if a complex were formed between the compound in question and calcium, most of the calcium would be bound in an inactive form and alternating additions of calcium and the compound would result in alternating increases and decreases in contractile amplitude. On the other hand if no such complex were formed and the response to calcium were specific then evidence of calcium toxicity should appear early as a result of an increased concentration of calcium ion in the medium. In the absence of compounds studied, a concentration of approximately 3 μmol/ml of calcium chloride in the medium proved toxic as evidenced by appearance of arrhythmias. Concentrations of calcium 2 or more times this value were required to produce arrhythmias when one of the compounds was in the medium (Table II). We are unaware of any report where an auricular depressant and calcium have been "titrated" in the manner described where there is more than enough calcium ion to cause toxic signs yet none are seen. Therefore, it appears that a part of the calcium has been converted to an inactive form and that the negative inotropic action of the compounds studied is related to their capacity to bind or inactivate calcium.

Although it would be of interest to relate dissociation constants of calcium complexes of these compounds to the capacity of calcium to reverse their toxic effects, there are such wide variations reported for constants by various investigators that it appears unwise, if not impossible, to attempt to do this. Ranges of reported values overlap to a considerable extent and the small differences observed correlate to a certain extent with small differences in calcium concentration required for toxic effects during "titration" experiments.

Summary. Various compounds of metabolic interest, both substrates (citrate, succinate, fumarate, l-malate, pyruvate, dl-lactate, acetate) and an inhibitor (malonate), produced a negative inotropic effect on isolated auricles of the rat. Of the intermediary metabolites the greatest degree of negative inotropic activity was exhibited by citrate and the least by acetate and lactate. Negative inotropic activity of these compounds did not appear to be significantly affected by temperature or addition of glucose to the medium. Results obtained following addition of calcium chloride to the medium, after effects of test compounds were established as well as results from "titration" experiments, suggest that the effects of metabolites and antimetabolite studied may be related, at least in part, to their capacity to form complexes with calcium.

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Parotid Gland Secretion Rate as Method for Measuring Response to Gustatory Stimuli in Humans.*† (25557)

HOWARD H. CHAUNCEY AND IRA L. SHANNON (Introduced by Robert T. Clark)
Tufts University School of Dental Medicine, Boston, Mass. and School of Aviation Medicine, USAF, Randolph Air Force Base, Texas

Four distinct submodalities, acid, salt, sweet and bitter, are generally acknowledged as comprising the gustatory sensation. These primary responses along with certain somatosensory components such as pain, temperature and touch contribute to the response aroused when receptor areas of oral cavity are activated by gustatory stimuli. Electrical recording of action potentials from single taste fibers (1) and response from total receptor field(2) provided a means of measuring nerve response to chemical stimulation of the tongue in animals. "Drop or sip" methods are generally employed to measure taste thresholds and sensitivity differentials in humans. Beebe-Center and Waddell(3) described a heteroqualitative taste scale using the subjective strength of 1.0% solution of sucrose, units being termed "gusts." Gust concentration curves were given for tartaric acid, sodium chloride, sucrose and quinine. Unfortunately, these measurements of human response to gustatory stimuli are based on subjective evaluations and are thus necessarily limited. Clearly, some other means of exploring taste in humans is needed before physiological mechanisms governing this sensation can be discovered. The present paper offers a new approach to quantitative measurement of the effect of gustatory stimuli.

Methods. One hundred thirty-eight male subjects between ages of 17 and 30 years, of sufficiently sound physical condition to qualify for military service, were utilized. *Stimuli*: The effect of each of 4 gustatory submodalities was determined using solutions of citric acid, sodium chloride, sucrose and quinine. Using wetted cotton applicator sticks, the solutions were applied by running wetted cotton around lateral edges of the tongue, followed by swabbing the entire dorsum of tongue from area delineated by the circumvallate papillae to the tip. A wall clock with sweep second hand was visible to all participants and supervisory personnel were available to insure that the subjects applied the stimuli in a proper manner and at specified intervals. *Secretory Rate*: Samples of parotid saliva, collected in tubes graduated to 0.1 ml were obtained by means of vacuum cups(4). Secretion rate (ml/min/gland) for each participant was determined by measuring time required to secrete a standard volume (5 ml) which permitted greatest accuracy in evaluating response to test stimuli. Response to different stimuli was quite variable and some individuals were unable to secrete the desired volume within a reasonable period. Since prolonged application of the stimuli caused undue discomfort or trauma to the subjects, (sodium chloride—drying of oral mucosa and lips; sucrose—nausea; quinine—retching; and citric acid—bleeding of the tongue and lips,) it was not feasible to continue testing for more than 4 hours. Therefore, maximum stimulation time, 20 minutes for sodium chloride and sucrose solutions and 15 minutes for citric acid and quinine solutions, was employed. If 5 ml was obtained before maximum time limit was reached the subject was permitted to terminate that sample and proceed to the next sample, to prevent glandular fatigue in persons who were rapid secretors. Secretion rate (volume collected/time of stimulation) for each individual and each variable was calculated. The results obtained, for each variable, were summed and mean rate for the group determined. Each experimental group received 4 to 8 test solutions. However, subjects were sampled only once for each variable tested. This procedure was employed after

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† The opinions asserted are those of the authors and are not to be construed as representing the views of the Air Force.

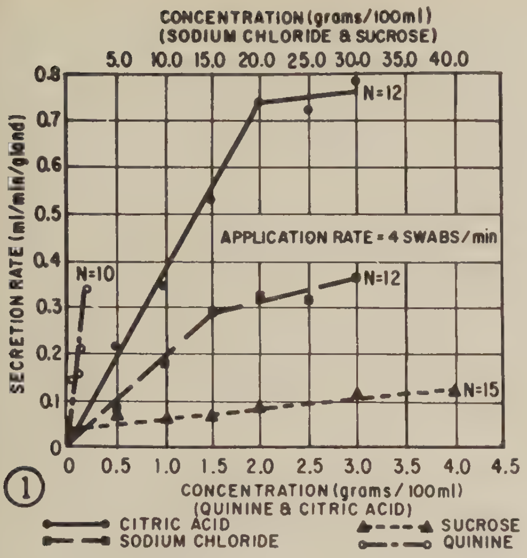
ascertaining in a pilot group (10 subjects) that both individual and group response to randomly chosen stimuli did not significantly vary when a given stimulus was applied several times within 4 hours. *Sampling*: A different group of individuals was employed for each series of experimental variables and the following standard procedure was established. After initial placement of vacuum cup a 10 minute acquaintance interval was given each subject. The secretion thus obtained was considered a *clearance sample* and was not used. Upon completion of the acquaintance session an *experimental sample* was collected. Since several successive samples were obtained from each individual, using both constant and variable stimuli, a 5 minute clearance sample was collected immediately prior to all other experimental samples. Participants were given a drink of water (room temp., 25°C) after collection of each experimental sample to make certain that the response measured was not affected by previously employed stimulus. Sampling was done both morning and afternoon without regard for possible effect of postprandial or diurnal variation. Three test conditions were utilized in determining parotid gland secretory response to the 4 gustatory submodalities. The effect of solution concentration, application rate (frequency of stimulation) and accommodation were investigated. Twelve experimental groups were tested. A control or sham group using only water was employed to determine response resulting from tactile sensation. Control values were thus subtracted from all experimental values, to give only the response arising from gustation.

Results. Effect of concentration: Range of concentrations of the 4 solutions used was chosen so that minimal concentration would be above absolute threshold for each sensation. Maximum concentration was selected either on a basis of solubility or on concentration suitable for continuous use without damaging oral mucosa or inducing illness. Concentrations were always given in ascending order. Throughout entire series of concentrations all subjects were able to discern increase in gustatory intensity from one solution to the next. A separate group of individuals was used for each of the 4 stimuli. Application rate was maintained at 4 swabs/minute.

Relationship between secretory response and solution concentration is illustrated in Fig. 1. Citric acid evoked a response which had a linear relationship with concentration up to a 2% solution. Secretion rate then appeared to have reached a maximum and broke sharply. A similar, but not so severe, break occurred with sodium chloride where a marked decrease in rate was exhibited at concentrations above 15%. In contrast, rate of secretion appeared to have a linear relationship with amount of quinine and sucrose in solution throughout entire series of concentrations. This is in accord with observations of Pfaffman(1) that frequency of response in any one gustatory fiber was increased by increase of stimulus intensity (concentration) only up to some limiting value. Above this limit, more intense stimuli produced only the same maximal response.

Effect of Application Rate: Intensity of response resulting from increasing rates of application is presented in Fig. 2. Solution concentrations were constant and rate of application (swabs/min) was increased throughout the series. Concentrations selected: citric acid—3%, sodium chloride—30%, sucrose—40% and quinine—0.15% induced maximal or near maximal stimulation. The results indicate that secretory response appears to be a curvilinear function of application rate (Fig. 2a). In the water series (control) secretion rate also increased as swab rate was raised, but here the relationship appeared to be a straight line function. When response (secretion rate) was plotted against logarithm of application rate, a linear relationship was established (Fig. 2b).

Accommodation. To ascertain if decreasing acceleration rate of response with increasing application rate was due to accommodation the following was undertaken. Five successive saliva samples were obtained, with exception of citric acid, using the same solution concentrations and collection procedures as for rate study. A lower concentration of citric acid (2%) was used because concentrations greater than 2% had a keratinolytic action when used over prolonged periods. Application rate was maintained at 4 swabs/minute; this approximated a mean rate of 4.7 swabs/min calculated from the range (0.5-12/



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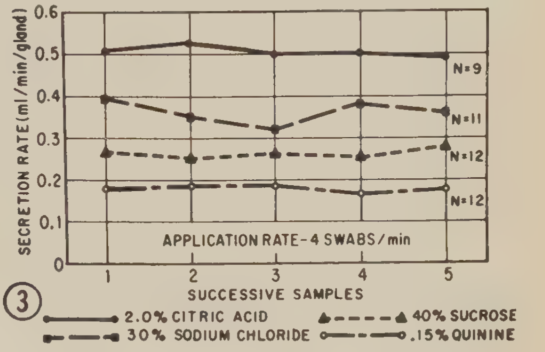

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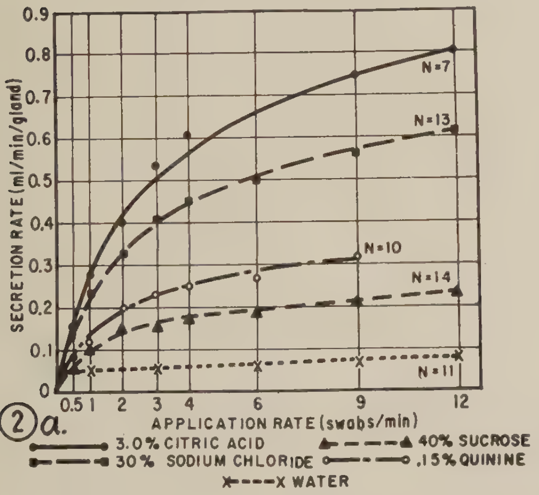

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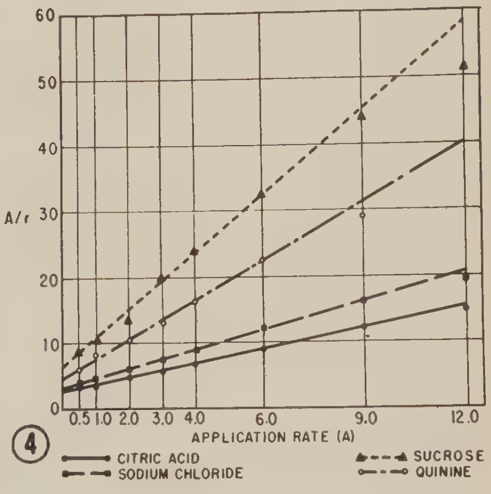

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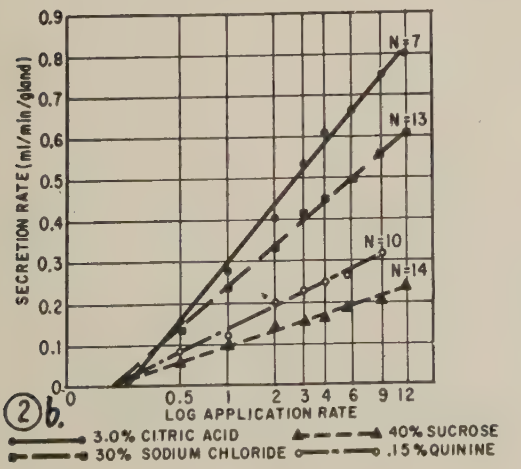

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FIG. 1. Effect of solution concentration on secretion rate. One group of subjects used for each of 4 stimuli. N equals No. of individuals/group. Saliva samples were collected successively and stimuli given in ascending order. Solution temp. 25°C.

FIG. 2a and 2b. Effect of application rate on secretion rate. N equals No. of persons/group. A separate group was employed for each of 4 test solutions and the water control. Temp. of solutions 25°C. Saliva samples were collected successively and application rate modified in ascending order.

FIG. 3. Effect of continuous stimulation on secretion rate. N equals No. of subjects/group. A different group was used for each test solution. Solutions maintained at 25°C.

FIG. 4. Determination of equilibrium constant and maximal response for 4 gustatory stimuli. Plot of 8 application rates (swabs/min.) against application rate divided by secretory response (ml/min./gland).

min) of the rate series. Fig. 3 shows that the response remained quite consistent over entire stimulation period (citric acid—80 min; sodium chloride—100 min; sucrose—130 min; quinine—130 min). Only sodium chloride and sucrose solutions exhibited a significant change in secretion rate between first and subsequent samples, in that paired data analysis of results indicated that there was a slight, but significant, difference in rate of secretion between samples 1 *vs.* 2, and 1 *vs.* 3. The difference between 1 *vs.* 4, 1 *vs.* 5 or among samples 2 through 5 was not statistically significant. With citric acid and quinine solutions, there were no statistically significant differences among any of the 5 samples. Thus it can be assumed that there was no measurable accommodation under imposed experimental conditions. Furthermore, these findings serve to reemphasize the observation noted in the pilot study, that subjects respond in a fairly consistent manner to a given stimulus.

Discussion. Plotting secretory response against stimulation frequency resulted in a curve fitting the equation of Michaelis and Menten. The method of Lineweaver and Burk(5) which rearranges the equation to a straight line function makes the relationship between response and affecting variable more obvious. This relationship can be expressed mathematically as $\frac{A}{r} = \frac{1}{R}(A) + \frac{K}{R}$, where A is affecting variable, r is observed response, R is maximum response and K is equilibrium constant of the entire reflex. By plotting A against A/r (Fig. 4) the slopes of resultant lines are equal to $\frac{1}{R}$ and the ordinate intercept is equal to $\frac{K}{R}$. The method of least squares was used to calculate slopes and ordinate intercepts. The R max and the gustatory-salivary reflex K for the 4 gustatory modalities are presented in Table I. The K for sucrose and quinine appear to be of similar magnitude and markedly differ from values obtained for citric acid and sodium chloride.

TABLE I. Maximum Response and Equilibrium Constant of Gustatory Stimuli.

Stimulus	R max, ml/min./gland	Equilibrium constant
Citric acid	.97	2.63
Sodium chloride	.71	2.21
Sucrose	.23	1.45
Quinine	.34	1.53

Due to the many problems which require further clarification, we can only speculate as to the meaning of these findings. Pfaffman (1) recording action potentials in cat chorda tympani and glossopharyngeal nerves was able to isolate 3 types of single fiber preparations. The first gave action potentials only when the tongue was stimulated with acid solution, the second responded only to acid and/or sodium chloride solution, while the third reacted to citric acid and/or quinine. Receptors for sugar were not usually found. Thus acid was a stimulus for all gustatory nerve endings while sodium chloride and quinine affected only certain types. In essence the 3 types were acid-salt, acid-quinine and pure acid fibers. In our investigation stimulation of the 3 primary modalities associated with fibers from the chordatympani which subserve, the sensation of taste in anterior two-thirds of tongue (acid, salt, and sweet) resulted in different responses as evidenced by equilibrium constants (K) for the gustato-salivary reflex. Although the bitter sensation is mediated, for the most part, through the glossopharyngeal nerve the equilibrium constant for quinine was similar in magnitude to that evidenced with sucrose. These findings appear to indicate that at least 3 different gustato-salivary reflex pathways are involved. Whether the difference lies merely in type of receptor ending stimulated, or depends also on different fiber types present in the gustatory nerve trunks cannot be discerned. However, it is believed that the procedure described herein offers a practical means of studying the physiology of human gustation and the mechanisms controlling salivary secretion.

Summary. Parotid gland secretion rate was employed for measuring human response to gustatory stimuli. Flow rate had a linear relationship to logarithm of frequency of stimu-

lation (swabs/min). Increasing concentration of gustatory stimuli resulted in increased rates of secretion. The response was linear throughout for sucrose and quinine, but with citric acid and sodium chloride it was linear only with lower concentrations. Mathematical expression of relationship between glandular secretion rate and frequency of stimulation permitted calculation of maximal response and reflex equilibrium constants for the various stimuli employed. Results indicated that at least 3 different gustato-salivary reflex pathways were involved.

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Urethan Induced Acceleration of Hexobarbital Metabolism.* (25558)

J. M. FUJIMOTO, D. E. BLICKENSTAFF AND F. W. SCHUELER
Dept. of Pharmacology, Tulane University School of Medicine, New Orleans, La.

Reduction and demethylation of certain azo dyes by rat liver microsomes have been increased by administration of such carcinogenic hydrocarbons as 3-methylcholanthrene and 3, 4 benzpyrene(1). The evidence is that induced enzyme synthesis occurs in these cases. Recently, increases in other hepatic microsomal enzyme activities have been obtained with several other classes of chemical compounds(2,3). The present article deals with data which suggest that urethan (ethyl carbamate) may similarly induce increases in activity of certain enzyme systems. Urethan is of particular interest owing to its cancer chemotherapeutic activity and carcinogenic activity.

Materials and methods. Sleeping times were measured in Swiss albino mice (20-35 g). Experimental groups were pretreated with urethan or 3-methylcholanthrene. After a definite time (in most cases 24 hours), these and control groups were given hexobarbital, sodium, and their sleeping times determined as duration of loss of righting reflex. Mice were considered asleep unless they could right themselves 3 times in one minute. Significance of the difference between sleeping times of control and experimental groups of mice was assessed by the "t" test. For determination of effect of urethan on hexobarbital body concentrations, the experimental mice were treated with urethan (1200 mg/kg, i.p.) and followed in 24 hours by hexobarbital sodium (150 mg/kg, i.p.). All drug solutions were of such concentration that .01 ml was given for each 1 gram body weight. Control mice received hexobarbital only. Determination of total body hexobarbital was done one hour after hexobarbital administration by homogenizing each mouse with 4 times its weight of water in a Waring Blender. Four ml of homogenate were extracted with chloroform by method similar to that of Axelrod *et al.*(4), except that the final NaOH extract of chloroform was immediately adjusted to pH 10.3 with $KHCO_3$ and read at 245 mμ in the Beckman DU spectrophotometer.

Results. In Table I pretreatment with urethan shortens hexobarbital sleeping time. Thus, if urethan is given 24 to 48 hours previous to hexobarbital, mean hexobarbital sleeping time is decreased from 41 to 31 minutes. This effect of urethan is largely gone by 72 hours. If the interval is only 12 hours between urethan and hexobarbital administration, sleeping time is prolonged beyond control levels owing possibly to persistence of CNS depressant effect of the urethan.

The most striking effects in shortening

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* Supported by an Institutional Research Grant Am. Cancer Society.

TABLE I. Urethan on Hexobarbital Sleeping Time (S.T.): Effect of Pretreatment Time.

Pretreatment with urethan*		Mean hexobarbital† S.T. ± S.E., min.	P
Control	(20)‡	40.9 ± 1.7	
12 hr	(20)	86.6 ± 6.3	.001
24	(19)	31.5 ± 1.1	”
48	(20)	31.1 ± 2.5	”
72	(18)	36.5 ± 2.5	.2

* 1200 mg/kg, i.p. † 150 mg/kg, i.p. ‡ No. of mice used.

hexobarbital sleeping time were observed when 1200 mg/kg urethan was administered 24 hours previous to hexobarbital (Table II).

In Tables I and II, a difference is noted in control sleeping times which occur with different batches of mice. Thus, controls were always tested with experimental group(s). That different batches of mice frequently yield variable results and therefore necessitate separate controls has been emphasized by others, for example Brown(5).

Since there existed the possibility that the vehicle for drug solution may have had effect, water and oil pretreated (24 hour, i.p.) mice were compared against untreated controls. These pretreatments had no significant effect (water, .01 ml/g, p = .8; oil, .01 ml/g, p = .9). As seen in Table III, shortening effect on hexobarbital sleeping time was obtained whether urethan was given i.p., s.c., or p.o.

The data for 3-methylcholanthrene indicate that it is qualitatively similar to urethan as regards effects on hexobarbital hypnosis. Pretreatment with 3-methylcholanthrene (30 mg/kg, i.p. in sesame oil; 24 hours) shortened hexobarbital (150 mg/kg, i.p.) sleeping time from 50.3 ± 3.3 to 37.7 ± 1.8 minutes (p = .01).

TABLE II. Urethan on Hexobarbital Sleeping Time: Effect of Dose of Urethan.

Urethan dose,* mg/kg, i.p.	Mean hexobarbital† S.T. ± S.E., min.	P
0	65.6 ± 2.0	
150	53.1 ± 3.8	.01
300	56.5 ± 5.2	.10
600	49.0 ± 6.0	.02
1200	39.0 ± 2.3	.001
2400	61.0 ± 5.2	.4

* Administration 24 hr before hexobarbital, 10 mice/group.
† 150 mg/kg, i.p.

The hexobarbital body concentrations in urethan-treated mice were lower than controls receiving hexobarbital alone. For the 9 urethan treated mice, the mean hexobarbital concentration was 36.4 ± 4.8 mg/kg as compared to a mean of 64.9 ± 7.3 mg/kg for the 12 control mice (p = .01). These data correlate well with data on the shortening of sleeping time produced by the urethan.

Discussion. The effects described suggest that urethan has the ability to increase in certain enzymes necessary for hexobarbital biotransformation. That is, the shortening of hexobarbital effect by urethan may be due to increased rate of hexobarbital biotransformation. It is unlikely that enhanced excretion of hexobarbital is responsible for the lowered hexobarbital levels. Except for initial urination and defecation at time of hexobarbital administration, little if any further urination or defecation occurs during the one hour period. Since the whole mouse is homogenized, any hexobarbital present in the bladder or gastrointestinal tract would be included in the total hexobarbital level. Since it is known that liver is the primary organ for hexobarbital metabolism(6) this effect of urethan is probably mediated in the liver. Since 3-methylcholanthrene increases liver microsomal enzymes(1,3), studies of mutual interactions of urethan, 3 methylcholanthrene and hexobarbital are indicated as interesting areas for further work.

TABLE III. Urethan on Hexobarbital Sleeping Time: Effect of Route of Administration of Urethan.

Pretreatment*		Mean hexobarbital†	P
Water, i.p.	(7)‡	68.5 ± 4.3	
Urethan, i.p.	(10)	45.8 ± 4.8	.01
Water, s.c.	(8)	66.7 ± 3.4	
Urethan, s.c.	(10)	41.5 ± 4.2	.001
Water, p.o.	(11)	63.5 ± 3.5	
Urethan, p.o.	(9)	40.9 ± 5.9	.01

* Administered 24 hr before hexobarbital. Water, .01 ml/g; urethan, 1200 mg/kg.
† 150 mg/kg, i.p.
‡ No. of mice used.

Summary. Urethan pretreatment shortens hexobarbital sleeping time and lowers hexobarbital body levels in mice. These results suggest that urethan increases rate of biotransformation of hexobarbital in mice.

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Serum Proteins of Rats after Partial Hepatectomy.* (25559)

S. G. A. ALIVISATOS, K. STERN, B. SAVICH AND L. LUKACS
Depts. of Pathology, Chicago Medical School, Mt. Sinai Hospital, and Mount Sinai Medical Research Fn., Chicago, Ill.

The assumption that the remarkable increase in mitotic activity associated with some stages of restoration of liver tissue after partial hepatectomy of rats is mediated by humoral factors has been supported by findings obtained after injection of serum from hepatectomized animals(1-3) and by results of parabiosis experiments(4-6). Changes in plasma and serum proteins of rats after partial hepatectomy have been investigated(7-11). During our studies on effects of injections of serum and serum fractions on mitotic activity and liver regeneration (to be presented later), we found it desirable to obtain base values correlating changes in mitotic activity with those of serum proteins following partial hepatectomy.

Methods. Rats of inbred Lewis strain, with average age of 68 days, were used. Weight of male animals was 212 ± 43 g, of females 182 ± 23 g. Blood specimens were obtained by cardiac puncture with needles and syringes coated with "Siliclad," to minimize hemolysis. Serum paper electrophoresis was carried out in Spinco model R instrument using 0.09 M veronal buffer at pH 8.65. Runs were carried out at room temperature with serum quantities of 0.013 to 0.015 ml and continued until satisfactory resolution of components was achieved. Average distance between distant ends of gamma globulin and albumin approximated 13 cm, requiring, as a rule, application of a potential difference of 120 volts for approximately 21 hours. Total protein was determined by digestion of an aliquot of serum and subsequent Nesslerization(12). At time of sacrifice, suitable tissue blocks were fixed in buffered formalin from which 6 μ thick sections were stained with Harris hematoxylin-eosin. In these slides mitotic activity was estimated by the method of Chalkley(13). Nuclei of liver cells were used as points of reference, and mitoses encountered as "hits" from scanning 500 nuclei were recorded for each section of liver tissue.

Results. In addition to 10 controls, 100 animals, evenly divided between males and females, were placed into 10 groups of 10 animals (5 males and 5 females), tested at one of 10 time intervals, the earliest being 6 hours after operation, the others as indicated in Fig. 1. Six animals of each group were subjected to partial hepatectomy according to procedure of Higgins and Anderson(14) while the remaining 4 were subjected to sham operations, *i.e.*, laparotomy and exposure of liver followed by closure of wound. Operations were carried out under ether anesthesia. With few exceptions surgery as well as sacrifice took place between 9 and 11 a.m., to avoid any fluctuations due to diurnal changes of mitotic activity(15). Animals were sacrificed at stated time intervals immediately after blood was obtained by cardiac puncture. Total body weights and weights of livers were recorded. After coagulation of blood specimens, serums were obtained by centrifugation for 5 minutes

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* Supported by grant from Ill. Div. of Am. Cancer Soc. and Helen Ecker Cancer Soc.

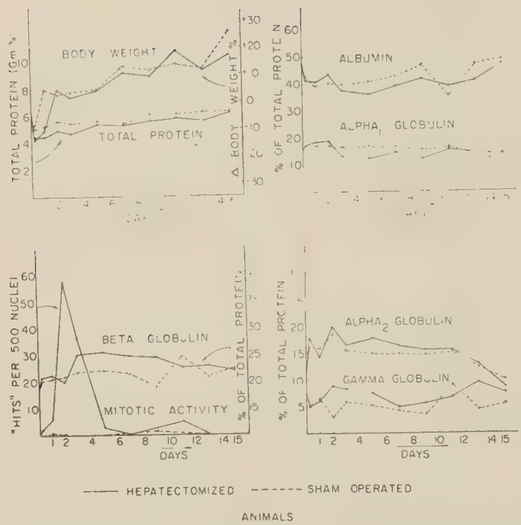

FIG. 1. Analytical data from hepatectomized and sham-operated rats. Each point represents mean of 6 partially hepatectomized or 4 sham-operated rats. Each of these groups comprised equal numbers of male and female animals.

at 600 x g. Preliminary hemoglobin determinations were carried out on all serum specimens; those containing more than 60 mg/100 ml were rejected and additional animals were tested. Determination of serum bilirubin indicated, as expected, moderate impairment of liver function after hepatectomy or sham-operation, with complete recovery not occurring during experimental period of 2 weeks.

Fig. 1 shows results of assays of total protein and protein fractions, as well as changes in body weight, and mitotic activity. The drop in body weight resulted most probably from dehydration. It was less pronounced in sham-operated animals and animals in both groups regained their original weights during observations of 2 weeks. Total serum protein values dropped immediately after hepatectomy and, to a lesser extent, also after sham operation.

The following comments may be made regarding changes in individual protein fractions:

(1) *Albumin.* This fraction reached its lowest concentration about 5 days after hepatectomy or sham operation and subsequently tended to rise toward normal levels. In general, the behavior of albumin paralleled approximately that of total serum protein. The changes were more pronounced after hepatectomy than after sham operation.

(2) *Globulins.* In hepatectomized as well as in sham-operated animals there was a rise in globulins, with hepatectomized animals affected to a greater degree. However, at least in the initial first 2 days this might have been due in part to changes in total blood volume. The most important changes were observed in the beta-globulin fraction. In hepatectomized animals this fraction tended to increase up to 60% beyond the values in controls, being most pronounced between third and ninth days after operation.

In sham-operated animals these changes were similar but of lesser magnitude. Other changes included an occasional separation of alpha$_2$-globulin fraction into 2 subfractions. This separation was not connected with presence of hemoglobin. In sham-operated animals a similar phenomenon was less pronounced.

Although changes in globulins tended to become less apparent toward end of experimental 2 weeks, their return to normal was not completed during this period.

In accord with well established data in the literature, mitotic activity reached its maximum between second and third day after hepatectomy and gradually fell to low levels between 5th and 7th day. A slight mitotic activity exceeding that occasionally found in sham-operated rats was observed in hepatectomized animals through the 11th day.

Changes in serum proteins described, on the whole, conformed to those reported previously (7-11), although in some instances differences in methodology make direct comparison difficult. Moreover, since these studies were designed to serve different purposes, comparison of changes in hepatectomized and sham-operated rats is possible in only one of them(7). On the other hand, detailed analysis of serum protein fractions during a prolonged period after hepatectomy is available only in another of these studies(11), but without consideration of sham-operated animals. In this study the most prominent change observed was a rise in beta-globulins which followed a similar pattern as in our work. It should be emphasized that in our own work, with the exception of mitotic activity, all changes observed—drop of body weight, total protein level and albumin, rise in globulin fractions, especially of beta-globulin—were present not only in hepatectomized but also in sham-operated rats with the former showing more pronounced changes than the latter. This makes it likely that these changes may, at least in part, be associated not only with liver regeneration but also with recovery from trauma and growth processes, such as those initiated by laparotomy incision.

Summary. Changes in serum proteins and mitotic activity in the liver of Lewis rats were studied after partial hepatectomy or sham operation, at intervals of 6 hours to 15 days. A rise of beta-globulin fraction roughly paralleled the rise in mitotic activity. Serum protein changes in sham-operated animals showed a similar trend but were less pronounced.

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Effects of Reserpine on Adrenal Responses to Nicotine.[*] (25560)

E. E. Bagwell, E. F. Woods and J. A. Richardson
Dept. of Pharmacology, Medical College of South Carolina, Charleston, S. C.

Small doses of nicotine injected into the thoracic aorta stimulate the adrenal medulla resulting in marked increases in levels of circulating catechol amines along with corresponding increases in heart contractile force (1,2). Our experiments were designed to study effects of previous reserpine treatment on (a) responsiveness of the adrenal medulla to chemical stimulation and (b) reactivity of the cardiovascular system to endogenously released catechol amines.

Methods. All experiments were performed on open-chest bilaterally vagotomized dogs. A total of 10 control and 25 reserpinized dogs were injected intra-aortically with test doses of 10 and 20 μg/kg of nicotine (administered as .01% solution of the alkaloid). Control dogs were anesthetized with 30 mg/kg pentobarbital sodium intravenously while reserpinized dogs, more sensitive to the anesthesia, were given only 15 to 20 mg/kg. Both groups were given supplemental doses as needed to maintain surgical state of anesthesia. Reserpine[†] was given intramuscularly and daily dosages varied from .05 to 1 mg/kg of body weight for 1 to 7 days. Plasma levels of epinephrine and norepinephrine were determined by modification(3) of the fluorimetric method of Weil-Malherbe and Bone(4). Blood samples were taken from indwelling polyethylene catheter placed in right femoral artery. The chest was opened by mid-line incision, and heart contractile force measured by suturing a strain gauge arch(5,6) directly to the surface

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[†] Serpasil generously supplied by CIBA Pharmaceutical Products, Summit, N. J.

TABLE I. Adrenal Medullary Stimulation Produced by Nicotine in Control and Reserpinized Dogs.

	Nicotine dosage, μg/kg	Heart force, % increase	Epinephrine, μg/l of plasma	Norepinephrine, μg/l of plasma
Control	10 (10)[*]	86.6	15.6	3.5
	20 (6)	137.5	46.6	8.8
Reserpinized	10 (18)	99.6	4.7[†]	2.5
	20 (16)	153.0	14.8[†]	4.7

[*] No. of dogs in parentheses. [†] Significantly different from control response ($P < .01$).

of right ventricle. Blood pressure was measured from the right carotid artery through a Statham transducer and recorded by strain analyzer and direct writing oscillograph from the Grass Instrument Co. Nicotine injections were made through polyethylene catheter passed up the left femoral artery into the thoracic aorta to a point just above origin of adrenal arteries. This enabled high concentrations of nicotine to pass into the adrenal glands and minimized the amount introduced into the general circulation. Doses of nicotine in these experiments (10 and 20 μg/kg) have been shown earlier to produce little or no effect when injected intravenously in intact animals or intra-aortically in adrenalectomized animals(2).

Results. Table I summarizes maximum mean percentage increases in heart contractile force and concentrations of epinephrine and norepinephrine during adrenal stimulation with nicotine. Ten μg/kg produced mean percent increase in heart force of 86.6% (S.E. ± 14.39) in normal dogs and 99.6% (S. E. ± 12.07) in reserpinized dogs. These differences were not statistically significant ($P > .1$). Blood pressure responses followed a similar pattern. However, on measuring plasma concentrations of epinephrine and norepinephrine from blood drawn at peak of response, the mean level of epinephrine was 15.6 (± 4.5) μg/liter of plasma in normal dogs while mean level in reserpinized animals was only 4.7 (± 1.4) μg/liter. This difference was highly significant ($P < .01$). Norepinephrine levels were not significantly altered ($P > .1$).

A similar pattern of response followed injections of larger doses of nicotine. Table I shows that increments in heart force produced by 20 μg/kg were not substantially altered by pretreatment with reserpine, while a marked differentiation may be noted in amounts of epinephrine present in plasma ($P < .01$). Again, norepinephrine concentrations were not significantly elevated.

Discussion. It is now generally known that reserpine depletes or reduces stores of catechol amines in such tissues as brain, arterial wall, heart, sympathetic nerve and adrenal medulla. Adrenals of the rabbit(7,8) and rat(9,10) are almost completely depleted of their amines, while the cat is more resistant and suffers only partial depletion with dosages of reserpine to 2.5 mg/kg(11). Maling *et al.* have more recently shown that the amine content of dog adrenals is also markedly reduced(12).

A question which has not been adequately studied is whether secretion of adrenal catechols, at rest and in response to stimulation, is diminished in proportion to reduction in tissue concentrations of these amines. Kroneberg and Schumann found that in rabbits the resting concentration of epinephrine in adrenal vein blood remained at 50% of normal when the adrenals were nearly completely exhausted by reserpinization(7).

Our experiments show that reserpine significantly decreases responsiveness of the adrenal medulla to chemical stimulation as indicated by smaller increments of plasma epinephrine in reserpinized dogs after nicotine injection (Table I). Since cardiovascular responses normally are proportional to levels of amines, it would be expected that changes in parameters such as heart force and blood pressure would also be less marked in reserpinized dogs. However, in our experiments, it appears possible that the cardiovascular system may be able to compensate for this diminution in adrenal responsiveness since heart force and blood pressure responses are not significantly different from those obtained in control dogs receiving the same doses of nicotine.

The mechanism by which this compensation occurs is not known. Consideration should be given to the fact that increased sensitivity of such parameters as heart rate and blood pressure to injected amines after reserpinization has been shown by several investigators (13,14,15,16). One may also speculate that the cardiovascular system becomes more sensitive to amines released by adrenal stimulation after reserpinization. However, a more detailed study needs to be made on sensitivity changes before conclusions can be drawn on this point.

Summary. Increments in plasma levels of epinephrine resulting from nicotine stimulation of the adrenal medulla are markedly reduced by reserpine. However, increments in heart contractile force and arterial blood pressure produced by adrenal stimulation, normally proportional to catechol amine levels, are not significantly altered following reserpine.

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Inhibition of Hexamminecobaltic Chloride, DNA Interaction by Sera of Patients with Disseminated Lupus Erythematosus.* (25561)

GERALD WEISSMANN† (Introduced by Lewis Thomas)
Dept. of Medicine and Study Group for Rheumatic Diseases, N. Y. Univ. College of Med., and Third (N.Y.U.) Medical Division, Bellevue Hospital, N. Y. City.

Evidence obtained by immunologic technics suggested that serum of patients with disseminated lupus erythematosus (DLE) contains several factors reacting with various nuclear materials. Holman and Kunkel(1) and Friou(2) indicated that gamma globulin of such sera is bound to both nuclei and nucleoprotein, while Miescher's studies(3) showed that nuclei of several sources can absorb from DLE sera the factor responsible for L. E. phenomenon, and also remove from such sera measurable amounts of gamma globulin. Classical precipitin curves have been obtained between protein-free desoxyribonucleic acid (DNA) and DLE serum(4) while latex particles(5) and red cells(6) coated with such material have been agglutinated by DLE serum. The reaction between highly polymerized DNA and the polyvalent cobalt salt hexamminecobaltic chloride ($Co(NH_3)_6Cl_3$) results in production of a clot of insoluble DNA. While studying this reaction, it was observed that DNA which had been treated with serum from patients with DLE, failed to clot, while normal serum had no such effect. The present paper deals with a simple method for demon-

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† Fellow of Arthritis and Rheumatism Fn., 1958-59.

stration of clot inhibition, and studies with a variety of sera are reported.

Materials and methods. Hexamminecobaltic chloride was prepared by the method of Bjerrum and McReynolds(7).[‡] Purity was established by nitrogen analyses and constancy of absorption spectrum upon reprecipitation, as well as by powder pattern X-ray diffraction. A 0.5% w/v aqueous solution was routinely employed, having absorbance of 1.004 at 470 μ in Beckman DU spectrophotometer. Such solutions were prepared weekly, and showed little loss of DNA clotting activity if stored at 4°C. Aqueous solutions of calf thymus DNA, stated to be highly polymerised and protein free[§] (500 μg/ml) were prepared and stored at 4°C. Immediately prior to use, aliquots were diluted 1:1 with distilled water. Such preparations were assayed for relative viscosity at 37°C. Relative viscosity was 4.45 ± 0.10 in Ostwald viscosimeters with water-flow time of 92 seconds. Constant viscosity after addition of $MgSO_4$ to 0.005 M was presumed to indicate absence of desoxyribonuclease (DNase). Histone of calf thymus origin and protamine sulfate[§] were prepared as 0.02% w/v serum solutions. Dried cartilage powder was prepared by homogenizing 2 g of beef nasal septal cartilage in a Vir-Tis 45 mixer at top speed in 250 cc of absolute ethanol. The residue was washed 3 times each with absolute ethanol and ether, and then dried *in vacuo* over $CaCl_2$. Whole dried calf thymus and guinea pig nuclei were obtained through the courtesy of Dr. Peter Miescher. Pancreatic DNase[‖], 100,000 units, was dissolved in 9 ml of 0.05 M tris (hydroxymethyl) aminomethane buffer pH 7.01. For activation of the enzyme, 1 ml of 0.1 M $MgSO_4$ was added, and resultant solution stored at 4°C prior to use. For routine testing, 0.4 ml of DNA (100 μg) were placed in small serology tubes, 0.5 ml of test serum was added with gentle agitation, and resultant mixture incubated at 37°C for 30 minutes. Thereafter, the tubes were brought to room temperature, 0.2 ml of hexamminecobaltic chloride solution added, and the tubes vigorously tapped with the forefinger, setting the solution spinning. In all "negative" sera, an easily visible, strand-like clot appeared immediately. This was graded $^{+}$ to $^{++++}$ depending on quantity of the clot formed. In positive sera, mainly from patients with DLE, no such clot formed. Instead, a slightly turbid solution resulted upon addition of the cobalt salt. All determinations were made in duplicate. Known inhibitory and normal sera were tested with each group of unknown sera. In some experiments clot inhibition was tested with various concentrations of DNA. To test whether inactivation of serum DNase would affect clot inhibition, 6 inhibitory sera were heated at 60°C for 30 minutes, prior to use. Patients diagnosed as DLE had signs and symptoms of that disease as well as positive L.E. cell preparations by the method of Hargraves(8). Patients diagnosed as probable DLE had similar signs and symptoms but did not have positive L.E. cell preparations. Sera were also obtained from 44 patients with rheumatoid arthritis, 2 of whom had positive L.E. cell preparations. The inhibitory effect of protamine sulfate or histone was tested by adding 0.1 ml of 0.02% solutions of these materials to 0.5 ml samples of normal serum mixed with 0.4 ml of standard DNA solution; the mixtures were incubated at 37°C, then 0.1 ml of a 1% w/v solution of cobalt salt was added. Absorption of sera was tested with nuclei or whole cartilage powder by incubating 50 mg (dry weight) of these materials for 4 hours at 37°C with 1.0 ml of serum; the mixture was centrifuged, and 0.5 ml of the supernatant was used for clot inhibition studies. For enzyme studies, 0.05 ml of DNase stock previously diluted 1:100 with 0.9% saline solution was incubated with 0.4 ml of 250 μg/ml DNA solution and 0.5 ml serum samples for 30 minutes at 37°C. Similar solutions were prepared with addition of sodium versenate (EDTA) 0.75 mg/ml to inactivate DNase as controls.

Results. Experiments summarized in Table I showed that in the presence of 0.5 ml of normal serum, at least 100 μg of DNA in aqueous solution were necessary before a clot appeared after addition of cobalt salt. Smaller amounts did not produce a visible

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[‡] Generously supplied by Dr. Maxwell Schubert.

[§] Nutritional Biochemicals Corp., Cleveland, O.

[‖] Lederle Laboratories, Pearl River, N. Y.

TABLE I. Hexamminecobaltic Chloride-DNA Clots in 0.5 ml of Serum (See Text).

μg DNA in 0.4 ml H_2O	DLE serum Loc.	DLE serum Ben.	Normal serum Nun.	Normal serum Rod.	Normal serum McC.
25	0	0	0	0	0
50	0	0	0	0	0
100	0	0	0	2+	3+
150	0	0	0	3+	3+
200	1+	0	3+	4+	4+
250	2+	3+	3+	4+	4+

clot. When similar experiments were performed in the presence of 3 DLE sera, no clot was visible with less than 200 μg. Using the technic described above, 20 of 31 DLE sera were positive, 5 of 12 probable DLE sera were positive, and 9 of 139 miscellaneous sera were positive (Table II). These included 4 sera from patients with convalescent hepatitis, 2 from patients given protamine (see below) and one each from patients with diagnoses of uncomplicated cirrhosis, penicillin allergy, and hepatic decompensation. Sera from 6 of 44 patients with rheumatoid arthritis were positive. Sera from patients with hypergammaglobulinemia due to sarcoidosis, cirrhosis, hepatoma, myeloma, macroglobulinemia or tuberculosis were negative.

When 6 positive sera were heated at 60°C for one half hour to inactivate serum DNase, no change in their inhibitory property was noted. Such heating did not cause negative sera to become inhibitory. Frequent thawing and freezing of some normal sera caused them to inhibit clot formation, while turbid sera interfered with a clear end-point. Sera showing an absorbance over .150 at 600 $m\mu$ in a Coleman Jr. spectrophotometer were not employed. Attempts to quantitate the reacting factor in DLE sera by dilution of whole sera were relatively unsuccessful. No inhibitory activity was observed at greater than 1:8 dilutions of several positive DLE sera.

TABLE II. Sera Inhibiting Hexamminecobaltic Chloride-DNA Clot Formation.

Diagnosis	DNA clot inhibition Positive	DNA clot inhibition Total tested	L.E. cell preparation Positive	L.E. cell preparation Total tested
Disseminated lupus erythematosus	20	31	31	31
Probable DLE	5	12	0	12
Rheumatoid arthritis	6	44	2	44
Misc.	9	139	0	9

Studies of inhibition of clot formation (Table III) indicated that DNA which had been depolymerised by pancreatic DNase no longer formed a clot on reacting with hexamminecobaltic chloride. This was true even in absence of serum, with saline used as diluent. When the action of DNase was inhibited by addition of EDTA, a clot readily formed. EDTA by itself had no effect upon clot formation or inhibition. When protamine sulfate or calf thymus histone were added to normal serum, clot inhibition similar to that observed with DLE serum was noted. If the specified concentrations were exceeded, these basic proteins formed DNA clots resembling those of cobalt salt in serum or saline. Incubation of positive DLE serum with whole calf thymus or guinea pig nuclei resulted in loss of inhibitory activity. To exclude a non-specific effect of polyanion, cartilage powder as a source of relatively insoluble chondromucoprotein was used in similar incubation experiments.

TABLE III. Hexamminecobaltic Chloride - DNA Clot Inhibition by Test Substances.*

Calf thymus DNA preparation in 0.4 ml H_2O		Whole serum DLE, Loc.	Whole serum Normal, Rod.
Polymerised	100 μg	0	3+
Depolymerised with DNase†	”	0	0
Polymerised (DNase + EDTA‡)	”	0	3+
		Absorbed with calf thymus or guinea pig nuclei	
Polymerised	”	2+	3+
		Absorbed with cartilage powder	
”	”	0	3+
		Protamine sulfate§ added to both sera	
”	”	0	0
		Calf thymus histone§ added to both sera	
”	”	0	0

* Clot formation graded + to 4+. Inhibition indicated: 0.

† Pancreatic DNase 10 units/ml + .005 M $MgSO_4$ for activation.

‡ EDTA 0.75 mg/ml to inactivate DNase by binding Mg^{++} ions.

§ 0.1 ml of 0.02% w/v solution.

No loss of inhibition resulted. In some DLE sera not giving a clot with DNA following addition of the cobalt salt, there was precipitate after 12 hours of standing at 4°C. This precipitate was no greater in those sera to which DNA *and* the cobalt salt had been added, than in those to which DNA alone had been. These precipitates, soluble in 1M NaCl, reacted strongly in capillary precipitin tubes with anti-human gamma globulin. Presumably these resembled the DNA-DLE gamma globulin precipitates described by Deicher *et al.*(4).

Discussion. There are 2 possible explanations for DNA clot inhibition following the addition of hexamminecobaltic chloride. Either the DNA could be depolymerised (as in the case of prior incubation with DNase) or the anionic groups of the polynucleotide could be linked by salt-like bonds to a factor in positive sera making these groups unavailable to the polyvalent cobalt cation. The latter possibility is supported by inhibition of DNA clot formation following addition of basic proteins like histone and protamine to normal serum. Failure of EDTA alone to block inhibition, and studies suggesting removal of the reactant from positive sera by incubation with calf thymus or guinea pig nuclei argue against the enzyme theory. Soluble DNA-protein complexes derived from calf thymus nuclei do not yield clots upon addition of hexamminecobaltic chloride, but do exhibit turbidities directly related to the amount of such complex present in solution. Since evidence from agglutination and precipitin studies indicates that globulin bound to nuclear extracts may well be a true antibody to such materials, it seems likely that formation of a soluble DNA-DLE serum complex prevents the polynucleotide from being precipitated in visible strands by cation. These observations resemble those of Lee(9), who used the agglutination of leukocytes by quinacrine as the phenomenon inhibited by DLE serum. The method described above deals with much more highly purified materials, has a more definite end-point, and seems of promise in assaying discrete serum fractions.

Summary. Normal sera do not inhibit the grossly visible clot formed by addition of hexamminecobaltic chloride to aqueous solutions of calf thymus DNA. The sera of approximately 66% of patients with DLE inhibit such clot formation. Prior treatment of DNA by active DNase also prevents clot formation, as does addition of histone or protamine to normal sera. The serum factor responsible for such inhibition is removed by prior incubation with whole calf thymus or guinea pig nuclei.

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Influence of Unbalanced Diets on Nitrogen Content and Enzymatic Activities of Hepatic Mitochondria of Rats. (25562)

NICOLA DIOGUARDI AND GIAN CARLO SECCHI (Introduced by G. Pozza)
Istituto di Clinica Medica Generale e di Terapia Medica, University of Milano

The aim of this work is to study whether an unbalanced dietary protein intake may cause changes in nitrogen content of the hepatic mitochondria. Previous observations(1) demonstrated that in normal rats fed protein deficient diet or fasted, some enzymatic activi-

TABLE I. Diet Composition.

	Normal balanced diet	Hypoprotein diet	Hyperprotein diet
	per 100 g		
Casein	18 g	4 g	40 g
Rice starch	68	82	46
Olive oil	10	10	10
Salt mixture (Osborne & Mendel)	4	4	4
Vit. D_2	62 units	62 units	62 units
" K	20 g	20 g	20 g
Thiamin	.4 mg	.4 mg	.4 mg
Lactoflavin	.8	.8	.8
Pyridoxine	1.2	1.2	1.2
Ca d-pantothenate	2	2	2
Nicotamide	12	12	12
P-aminobenzoic acid	120	120	120
Biotin	20 g	20 g	20 g
Vit. B_{12}	4 μg	4 μg	4 μg
Folic acid	40	40	40
Inositol	60 mg	60 mg	60 mg
Choline	120	120	120

ties of the hepatic mitochondria/mg of mitochondrial nitrogen varied inversely with protein intake. These data led to the hypothesis that protein deficiency may cause a reduction of nitrogenous material in mitochondria and that consequently it may not be always advisable to relate enzymatic activity of mitochondria, to their nitrogen content, as is generally done. In our work mitochondria were counted to study the effect of fasting and of hypo- and hyperprotein diets on nitrogen content of each mitochondrion and on concentration of several enzymes/single mitochondrion and/mg of mitochondrial nitrogen.

Materials and methods. Male Wistar rats about 90 days old were fed 3 different food mixtures (Table I). All animals received a weekly supplement of 350 units of Vit. A. At end of experiment, the animals were fasted 12 hours and decapitated. The liver was quickly removed and the homogenate prepared at 0°. Mitochondria were separated from homogenate according to Schneider(2) in a solution of 0.25 M sucrose and 0.01 M ethylenediaminotetraacetic acid (EDTA), and washed 3 times in the same solution. The following activities were investigated: cytochrome-oxidase(3), succinic-oxidase(3), beta-hydroxybutyric-oxidase(3), ATPase(4). Nitrogen was determined by Nessler's reagent. Mitochondria

TABLE II. Nitrogen Content and Enzymatic Activities.

Diets	No. exp.	Nitrogen/mitochondrion, mg × 10^{-11}	Cytochrome oxidase		Succinic oxidase		Hydroxybutyric oxidase		ATPase	
			μl of O_2/hr						μmoles P/hr	
			Mitochondrion × 10^{-8}	mg N	Mitochondrion × 10^{-8}	mg N	Mitochondrion × 10^{-8}	mg N	Mitochondrion × 10^{-8}	mg N
1. Normal	15	3.10 ± .12	5.83 ± .35	2000 ± 122	2.50 ± .32	700 ± 53	2.69 ± .34	930 ± 69	6.0 ± 1.0	160 ± 13
2. Hypoprotein	15	1.75 ± .08	4.50 ± .30	3200 ± 186	1.30 ± .20	1110 ± 124	1.16 ± .24	860 ± 65	4.0 ± .6	182 ± 19
3. Fasting	13	.92 ± .04	2.87 ± .39	3100 ± 218	.80 ± .17	1300 ± 123	1.00 ± .24	1500 ± 155	3.0 ± .5	288 ± 33
4. Hyperprotein	13	3.11 ± .11	10.20 ± .65	3320 ± 190	2.45 ± .50	720 ± 48	2.58 ± .4	950 ± 71	6.0 ± 1.5	160 ± 14
Statistical significance										
1-2		P <.05	P <.05	P <.001	P <.05	P <.05	P <.001	P >.05	P <.001	P >.05
1-3		P <.001	P <.001	"	P <.001	P <.001	"	P <.001	"	P <.001
1-4		P >.05	"	"	P >.05	P >.05	P >.05	P >.05		

were counted in a counting chamber using a phase-contrast microscope.

Results. Table II shows results. The hypoprotein diet caused marked reduction of nitrogen content without conspicuous decrease in enzymatic activities of single mitochondria. Fasting caused a very distinct decrease of nitrogen content with simultaneous decrease of enzymatic activities of single mitochondria. However, since decrease in enzymatic activities was smaller than decrease in nitrogen content, activity/mg of mitochondrial nitrogen appears to have increased. The hyperprotein diet did not cause significant changes in mitochondrial nitrogen content. On the other hand, it caused marked increase of cytochrome-oxidase activity/mitochondrion and/mg of mitochondrial nitrogen. Other enzymatic activities studied did not change significantly.

Discussion. It is obvious that under suitable experimental conditions the nitrogen content of hepatic mitochondria may undergo conspicuous variations and that by means of suitable protein deficient diets it is possible to reduce mitochondrial nitrogen content without reducing activity of certain enzymes significantly.

The results of these experiments suggest that hepatic mitochondria contain 2 nitrogen fractions with different functional characteristics: an enzymatically inert labile fraction and an enzymatically active one. Protracted fasting causes greater decrease of mitochondrial nitrogen and a much more pronounced fall of enzymatic activities, suggesting that, in this condition, there is significant loss of enzymatically active nitrogen. This appears to be bound more firmly to the mitochondrial structure than the enzymatically inactive fraction, which can be reduced by protein deficient diet. The increased cytochrome-oxidase activity observed following the hyperproteic diet suggests that the portion of the enzyme which under normal conditions is enzymatically inactive, may become activated. Our results further suggest that in experimental conditions able to change the nitrogen content of mitochondria, the data concerning enzymatic activities should be reported in terms of single mitochondria, rather than in terms of mg of nitrogen, as it is usually done.

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Blood Thrombocyte Levels after Administration of Equine Estrogens. (25563)

D. FIRAT AND L. STUTZMAN (Introduced by Julian L. Ambrus)
(With technical assistance of F. Szymanski and G. Ziehn)
Division of Medicine, Roswell Park Memorial Inst., Buffalo, N. Y.

Intravenous estrogens in uncontrolled studies have been reported beneficial in cases of hemorrhage of various types including neonatal(1), uterine(2,3), post dental extraction (4), nasal and post adenoidectomy(2,5), as well as rectal, gastric, and subarachnoid bleeding. However, the mechanism of reported hemostatic effect has not been adequately explained. In dogs, Johnson(6) found elevation of prothrombin and Ac-globulin as well as decrease of antithrombin following intravenous Premarin.* Increased levels of prothrombin and prothrombin-conversion accelerators have been found in late pregnancy(7). Thrombocytosis following estrogen injection was reported by Menger(2) but not seen by Jacobson(8) or Whittington(4). A sharp elevation of thrombocyte counts occurs just before ovulation(9,10), coinciding with the peak in estrogenic hormone levels. We have compared platelet counts before and after single intravenous injections of 20 mg of Premarin.

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* "Premarin," available from Ayerst Lab., consisting of a mixture of equine conjugated estrogens.

Method and materials. Nine patients with lymphoma or chronic leukemia, who had thrombocytopenia either as manifestation of the disease or secondary to chemotherapy, and 5 patients with normal platelet counts were studied. The experimental period lasted 4 days. Each patient received 5 cc of physiological saline intravenously at 10 a.m. on first and fourth days. On second and third days, 20 mg (5 cc) of Premarin was given intravenously at 10 a.m. Venous blood was withdrawn for platelet counting immediately before, and 2 and 4 hours after, these injections. Patients ate lunch soon after the 2 hour sample was obtained. In a few instances, patients could not be studied over 4 day periods and one of the control days was omitted. Siliconized syringes, needles, and blood containers were used. Syringes were moistened with heparin solution for anticoagulation. Platelet counts were performed in duplicate within 1 hour of drawing blood samples by the same technician, using a modification of hemocytometer with phase microscopy method of Brecker, Scheiderman and Cronkite(11). Reproducibility of this method was more satisfactory than with indirect methods for counting platelets. Hematocrit and WBC determinations were performed on all samples as additional check of technic.

Results. Patients with initial platelet counts under 100,000/cmm (ranging from 12,700 to 80,000/cmm) and those with counts over 100,000/cmm (ranging from 182,000 to 360,000/cmm) were grouped separately. Reproducibility of counts was satisfactory. There were no consistent variations between the 2 control days or the 2 treatment days. Percentage deviations from pre-injection values after 2 hr and 4 hr were calculated. Mean changes and their standard errors, for both groups of patients, are shown in Fig. 1. It is evident that there was no significant difference between effects of saline and Premarin.

No significant variations in white blood cell counts or hematocrit were seen.

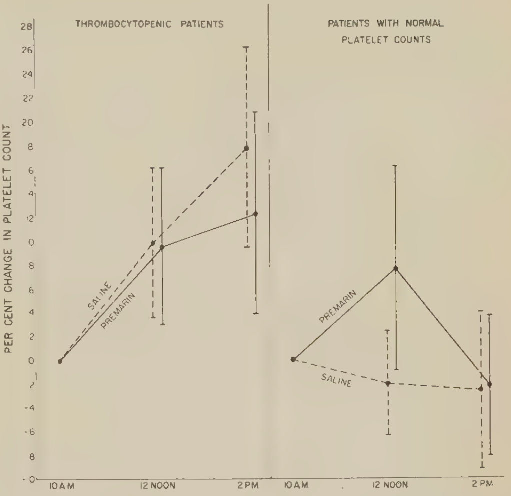

FIG. 1. Response of thrombocytopenic patients and patients with normal platelet counts to intrav. normal saline and Premarin inj. Vertical bars represent stand. errors of percentage changes.

On control days of saline injections, a slight increase of platelet counts occurred in the thrombocytopenic group, but no change was noted in the group with normal platelet counts. On treatment days, platelet counts increased slightly during first 2 hours after Premarin injection. This elevation was maintained for the 4 hour study period in the thrombocytopenic group but returned to resting values in patients with normal counts.

Change of platelet counts from 10 a.m. to 2 p.m. after saline injection may reflect part of a diurnal variation, since such variations of thrombocytes have been observed. Matis and Gross(12) found a significant increase in platelet counts during the late afternoon in subjects on normal routines. On the other hand, Ivanitzky and Klimova(13) observed a decrease in numbers of circulating platelets under these conditions, with disappearance of this variation during a 24 hour fast. The variations in the present study appear to parallel those described by Matis and Gross; however, the changes in Fig. 1 are not statistically significant at the 5% level of probability. Although diurnal variation is suggested, our study revealed little or no change in thrombocyte counts from day to day. Since the life span of platelets is between 3 and 10 days (14), a day to day increase in thrombocyte counts would have been expected if Premarin mediated an acute release of new platelets from bone marrow.

Summary. Administration of mixed natural estrogens (Premarin) intravenously to 5 patients with normal platelet counts and to 9 patients with thrombocytopenia did not produce a significant change in thrombocyte counts of peripheral blood, as compared to saline injections in the same patients. Premarin appears to be of no value in controlling bleeding due to thrombocytopenia.

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In vitro Effect of *Vicia faba* Extracts upon Reduced Glutathione of Erythrocytes. (25564)

DERYCK G. WALKER AND JAMES E. BOWMAN (Introduced by James A. Halsted)
Dept. of Biochemistry, Faculty of Medicine, University of Shiraz, and Dept of Pathology, Shiraz Medical Center, Nemazee Hospital, Shiraz, Iran

Glutathione (GSH) stability test of erythrocytes and other technics indicated that the basic abnormality in persons sensitive to primaquine, other drugs, and to fava bean (*Vicia faba*) is due to the same genetically determined erythrocytic defect(1). Destruction of GSH both *in vitro* and *in vivo* is caused by primaquine, phenylhydrazine, aniline, hydroxylamine, nitrofurantoin, α and β naphthol, α and β naphthoquinone, and certain Vit. K derivatives(1). Although extracts of *Vicia faba* have been reported as not destroying red cell GSH *in vitro*(2), in the present study certain effects of *Vicia faba* extracts on GSH of erythrocytes have been observed.

Materials and methods. Preparation of *Vicia faba* extract (VFE) was based upon the technic of Dunsford and Bowley(3) for plant hemagglutinins. Fresh beans were soaked in excess of water at 18 to 20°C for 12 to 18 hours. Remaining water was then drained off. The beans were macerated in Waring blendor for about 1 minute. Two volumes of 0.9% sodium chloride solution were added to 1 volume of macerated material. After mixing and standing 1 hour, the mixture was filtered through several layers of fine mesh gauze. The filtrate, which still contained a small amount of sediment, was collected into convenient lots and stored at –20°C until required. Full activity remained for at least 3 months. Three volumes of blood were added to 1 volume of standard ACD solution. The hematocrit of these suspensions was measured. For preliminary experiments, blood from persons of known response to GSH stability test was used. Survey bloods were taken from random selection of members of the Mamassani tribe of Southwestern Iran. Reduced glutathione (GSH) measurements and GSH stability test with acetylphenylhydrazine (APH) were performed as described by Beutler(4). For testing effect of VFE, 0.5 ml of the extract was added to 1

TABLE I. Effect of APH and VFE on 113 Blood Samples from Members of the Mamassani Tribe.

	No. of subjects	Treatment	Mean GSH*
Nonsensitive males	55	None	61.2 ± 10.7
		APH	60.2 ± 12.1
		VFE	51.5 ± 12.5
Sensitive males	14	None	42.0 ± 7.4
		APH	6.9 ± 3.8
		VFE	14.1 ± 2.7
Nonsensitive females	41	None	72.8 ± 16.8
		APH	66.0 ± 16.7
		VFE	56.9 ± 12.5
Sensitive females	3	None	63.2 ± 12.3
		APH	16.8 ± 4.9
		VFE	26.6 ± 6.3

* GSH in mg/100 ml red blood cells ± stand. dev.

ml of oxygenated blood and incubated at 37°C in water bath for desired time with occasional shaking. Since volume of extract used was not in excess of 1 ml, no attempt was made to change volumes of reagents required for hemolysis and protein precipitation from those normally used in GSH stability test. However, calculations were corrected for increase in volume after protein precipitation.

Results. Preliminary experiments indicated that when blood from sensitive persons was incubated with VFE there was a fall of GSH in red blood cells. A lesser fall in GSH was observed with blood from nonsensitive individuals. Virtually all GSH decrease occurred within 10 minutes. No hemolysis was noted. Dilution of VFE diminished its effect on GSH so that, with 1:10 dilution, distinction between sensitive and nonsensitive bloods became negligible. *In vitro* effect was further shown by performing parallel incubations with APH for 2 hours and VFE for 30 minutes on 113 blood samples (Table I). VFE had a lesser effect on GSH of erythrocytes of sensitive persons than APH and a greater effect than APH on nonsensitive erythrocytes. Using less than 30 mg GSH/100 ml rbc as a criterion of sensitivity(5), the 2 agents gave anomalous results in 3 cases. Two males, nonsensitive with APH (post-incubation levels of 42.1 and 42.7 mg GSH/100 ml rbc), gave values after incubation with VFE of 28.3 and 28.8 mg GSH/100 ml rbc respectively. One female, sensitive with APH (post-incubation level of 22.4 mg GSH/100 ml rbc), had 33.7 mg GSH/100 ml rbc after incubation with VFE. Finally, extracts of beans boiled for 1½ hours were prepared as for fresh beans. There was no significant effect on GSH with these extracts.

Discussion. The mechanism of hemolysis on exposure to fava bean still is not explained (2). This bean is widely grown and commonly eaten in Iran. We have shown(6) that approximately 8% of Moslems in Iran are sensitive with the GSH stability test; yet fewer cases of favism are seen than might be predicted from this figure. Many of our sensitive subjects eat the bean regularly without noticeable effect. Furthermore, persons with symptoms of favism gave histories of previous ingestion without illness(2). Since favism usually follows ingestion of fresh beans(7), their preparation may influence the nature and severity of hemolysis. Although we showed a direct *in vitro* effect of VFE on erythrocytes GSH, we can not exclude evidence for allergy in addition to the genetic erythrocytic defect(2). Determination of the chemical nature and properties of the agent in our extract may clarify the pathogenesis of favism.

Summary. A heat labile extract of *Vicia faba* was prepared which, like APH, lowered the reduced glutathione of erythrocytes of sensitive individuals.

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Occurrence of Hemorrhagic Centrolobular Liver Necrosis in Protein Deficient Rats.* (25565)

MAX WACHSTEIN AND RUTH SCHWARZ
Dept. of Pathology, St. Catherine's Hospital, Brooklyn, N. Y.

During study of the pancreas of rats on synthetic but otherwise complete protein-deficient diets, changes in several organs were observed, particularly occurrence of centrolobular necrosis in livers of some animals. The important relationship of dietary liver cell necrosis for development of permanent liver damage has been recently emphasized by Bras, Goldblatt and György(1). For this reason, and also because central hemorrhagic necrosis is quite commonly found in livers of animals given dl-ethionine, a presumed methionine antagonist, a more detailed investigation in the incidence of this lesion in complete and partially protein-deficient rats was made. The influence of added cystine and methionine was also studied.

Material and method. Young male rats of Wistar strain weighing 120-170 g were divided into 9 groups. They were given a basic protein-free diet which contained corn starch 70%, vegetable oil 10%, cellulose 15%, cod liver oil 1% and salt mixture U.S.P. No. XIV. Vit. supplements/kg of food were added as follows: Vit. A concentrate 20,000 U, Viosterol 11,000 U, Inositol 110 mg, alpha-tocopherol 110 mg, choline chloride 1.65 g, Menadione 49 mg, para-amino benzoic acid 110 mg, niacin 100 mg, riboflavin 22 mg, pyridoxine 22 mg, thiamine 22 mg, calcium pantothenate 66 mg, biotin 0.44 mg, folic acid 2 mg, Vit. B_{12} 0.03 mg. Group 1 received the basic protein-free diet while Groups 2 to 8 received various food supplements in addition (Table I). In Group 9, 60% of vegetable fat (Crisco) was substituted for equivalent amount of starch in diet which contained 7.5% casein. Rats were weighed and sacrificed after 43 to 55 days. Slices of liver, kidney and pancreas were fixed in Bouin's solution as well as in 10% formalin and stained with hematoxylin-eosin. Iron and fat stains were done when indicated. Necrobiotic changes which occurred in many livers were graded as follows: ± when only isolated cells in close vicinity to the central vein were involved, and +1 when necrosis of from 1 to 2 rows of cells occurred. More extensive lesions were classified as +2. The severest degree of central necrosis involved to one-half of lobe was considered +3.

Results (*Table I*) - *Group 1.* Typical microscopic changes of protein depletion as described by various investigators(2,3,4) were seen in the liver of 60% of the rats. Liver cells appeared large and swollen. The cytoplasm appeared vacuolated and rarified due to considerable decrease of basophilic granules, while cell membranes stained prominently. The nuclei lost their vesicular appearance and became somewhat hyperchromatic. The sinusoids were narrow and often completely compressed. Fatty changes were also frequently observed. In addition, a centrolobular necrosis graded +1 to +3 was seen in about ½ of all animals. This necrosis was accompanied by fresh hemorrhages in central areas. The extension of such hemorrhages was in most instances proportional to necrotic damage. However, some livers presented extensive hemorrhages with minimal necrosis and vice versa. Infiltration by lymphocytes and large mononuclear cells was sometimes observed in necrotic areas. Occasionally iron containing histiocytes was seen, obviously indicating older hemorrhages.

Group 2. Liver cells showed a similar degree of rarifaction of the cytoplasm, as observed in Group 1. There was no significant difference in regard to incidence and severity of the centrolobular necrosis, since +1 to +3 lesions were encountered in 55% of the animals.

Group 3. Rarifaction of cytoplasm was seen in 2 out of 8 animals. Central necrosis judged +1 to +2 was present in 2 animals. No +3 lesion was observed in this group. Mild fatty changes were present in 1 animal as minute fatty droplets without zonal distribution.

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* Supported by a grant from Vitamin Fn., N. Y.

TABLE I. Summary of Findings in Groups of Rats on a Protein-Free Diet with and without Various Supplements.

Group	Diet	No. of rats	Days on diet	Initial wt, g	Wt changes, g	No. of rats showing necrosis 0	±	1+	2+	3+	% of rats showing 0 & ± lesions	1+ to 3+ lesions
1	Basic protein-free	34	43–55	120–170	All lost 45–90	10	9	5	9	1	56	44
2	*Idem* + 2% casein	22	”	120–160	*Idem* 35–85	6	4	7	5	0	45	55
3	” + 4% ”	8	49–55	130–160	” 30–50	6	0	1	1	0	75	25
4	” + 7.5% ”	13	45–55	140–170	12 lost 10–30 1 no change	6	6	1	0	0	92	8
5	” + 1% methionine	14	46–55	”	All lost 40–90	4	2	3	4	1	43	57
6	” + 2% casein + 1% methionine	14	”	150–160	*Idem* 50–70	1	2	3	5	3	21	79
7	” + .5% cystine	14	46	160–170	” 50–80	1	0	4	5	4	7	93
8	” + 2% casein + .5% cystine	7	45–55	140–160	” 20–60	0	1	3	3	0	14	86
9	7.5% casein diet 60% fat	14	”	150–160	5 lost 10–20 6 unchanged 3 gained 40	4	2	5	3	0	42	58

Group 4. Central necrosis was almost completely absent since only 1 out of 13 animals presented a +1 lesion. Typical cytologic changes of protein depletion were seen in only 1 case. Fatty changes were minimal in 2 rats.

Groups 5 and 6. These 2 groups can be discussed together as the effects on these diets were similar. Typical protein depletion changes in liver cells were observed in most animals. Fifty-seven and 79% respectively of livers showed +1 to +3 central necrosis. Fatty changes ranged from minute fatty droplets in occasional liver cells with or without zonal arrangement to severe fatty infiltration as large droplets in many liver cells, mainly affecting cells in periportal areas.

Groups 7 and 8. No significant difference was seen in these 2 groups. Typical changes of protein depletion of liver cells were seen in both groups in 30% of the animals. Centrolobular necrosis graded +1 to +3 was encountered in 93% of rats in Group 7 and 86% in Group 8, accompanied by extensive recent hemorrhages. Fifty % of animals in both groups showed also histiocytes containing hemosiderin pigment in the centrolobular areas. Mild fatty changes were seen in few animals, located chiefly in periportal and mid-zonal areas.

Group 9. Fifty-eight % of rats presented central necrosis graded +1 to +3 with extensive hemorrhages. Fatty changes of liver cells were severe and observed in all animals. These fatty changes were limited to periportal liver cell cords but extended in several instances into mid-zonal areas. In contrast, cytoplasmic vacuoles of liver cells due to protein depletion present in all instances were also observed in central areas.

Discussion. On complete protein deficient diet liver cells show rarifaction of cytoplasm due to disappearance of their basophilic granules, the so-called protein storage particles of Berg which are thought to be ribonucleic acid aggregates(4). In these livers increase in glycogen and lipids has been reported(4,5). In our material fatty changes varied from a very slight to a fairly marked degree. Rarifaction of cytoplasm and fatty changes were not essentially influenced by addition of 2% casein while a supplement of 4% casein led to distinct improvement in the microscopic appearance. When the diet contained 7.5% casein, liver cells were almost normal, provided fat content was not unduly raised. If 60% of carbohydrates were replaced by equal amount of fat in the diet, rarifaction of cytoplasm, associated with severe fatty changes, was again noted(6).

Centrolobular necrosis was present in rats on complete protein-free and 2% casein diet. There was a protective influence of 4, and more marked of 7.5% casein. Addition of methionine or cystine did not prevent the lesions. Cystine even aggravated severity of centrolobular necrosis. This is probably due to a direct necrotizing effect of cystine(7,8).

Few investigators have studied livers of rats on severely protein-deficient diets. Central necrosis was not found by Kosterlitz in rats on a protein-free diet to 28 days(4), or by Jaffe *et al.*(9), who kept their rats to 111 days on a diet containing only 2% protein. Centrolobular necrosis was reported in some experiments containing 4 to 6% casein(10,11). However, these diets were deficient in Vit. E. György and Goldblatt(12,13), fed as much as 18% casein, and observed a variety of hepatic lesions including central and mid-zonal necrosis. Addition of yeast or Peter's eluate, however, completely prevented occurrence of these lesions. Massive necrosis of liver cells seen when highly purified casein or certain yeast proteins are fed, is obviously of different etiology, since it can be prevented by either of 3 well defined factors, including Vit. E(14). The very occasional occurrence of focal liver cell necrosis in a diet containing 9% casein, all essential vitamins and alpha-tocopherol reported by Herrera *et al.*(15) is in agreement with our findings of occasional necrosis in rats consuming sub-optimal amounts of protein. Centrolobular necrosis in combination with hemorrhages in a substantial percentage of our animals on protein deficient diets is of considerable interest in view of the microscopically similar centrolobular necrosis following administration of dl-ethionine. It is generally assumed that dl-ethionine acts as a methionine antagonist, since addition of equimolecular amounts of methionine to the diet completely prevents ethionine lesions. Such a favorable effect of methionine was not seen in protein deficient animals. Other hepatic lesions which regularly occur in animals given ethionine, *i.e.*, characteristic proliferation of "ductular cells" and of regenerative changes were not seen in our animals(16,17). This again indicates that the ethionine lesion is probably of a complex nature and not only due to a conditioned methionine deficiency(18). Rats force-fed diets deficient in certain amino acids developed fatty infiltration, predominantly periportal in distribution, but not necrotic changes(19,20).

Central necrosis has been described in totally starved mice(21). It is, however, unlikely that the changes in our rats were due to starvation, since they were also seen in rats given 7.5% casein and 60% fat. The majority of animals in this group lost no weight and some even gained weight.

We previously described atrophic lesions of the pancreas on protein deficient diets and found that addition of 2% casein was without influence, while 4% casein or 1% methionine had a marked beneficial effect(22). In the present investigation, we again found considerable improvement on the 4% casein diet. However, we were unable to reproduce our previous finding of a beneficial effect of a methionine supplement.

Summary. On a synthetic protein-free diet containing all other essential nutrients with or without a 2% casein supplement, the livers of young male Wistar strain rats show centrolobular necrosis, often associated with hemorrhages in about 50% of animals. Incidence of these lesions decreases when casein content of diet is increased. Addition of 1% methionine is of no influence, while 0.5% cystine often aggravates the severity and incidence of centrolobular necrosis. The findings are discussed with reference to hemorrhagic centrolobular necrosis which occurs frequently after administration of ethionine, a supposed methionine antagonist.

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Effect of Krebs-Ringer Medium on Muscle Contraction *In situ* in Adrenalectomized Albino Rats. (25566)

SAUL M. SCHANBERG* AND I. A. MACCHI† (Introduced by G. P. Fulton)
Dept. of Biology, Clark University, Worcester, Mass.

Although it is well established that adrenal cortical insufficiency is accompanied by reduced ability for sustained muscle performance *in vivo,* underlying mechanisms are not clearly understood. Tensile(1,2), electrical (2), or biochemical(3) properties of resting muscle are apparently not sufficiently altered in adrenal insufficiency to be implicated in any major way as direct targets of corticoid action. Nevertheless, corticoids normally may act directly on contractile processes to a limited extent(4). Although weight of evidence (5,6,7,8,9) suggests that impaired muscle performance *in vivo* in adrenal insufficiency is related to well known accompanying circulatory and cardiovascular disturbances(10,11), it is not certain whether these contribute directly by acting on contractile processes or indirectly by interfering with circulatory adjustments normally occurring under physiological demands for sustained muscular activity. Whatever the underlying physiological mechanism, immediate cause of impaired muscle function may be consequential to inadequate transport of metabolic substances vital to sustained muscle activity or to inadequate removal of toxic metabolites. The immediate importance of circulatory factors is suggested by the work of Ramey, Goldstein, and Levine (5) who have shown that differences in work performance *in vivo* of intact and adrenalectomized rats fatigued by swimming are not reflected in diaphragm and oblique abdominal muscles isolated to nutrient medium. However, in these studies differences in muscle work performance prior to isolation were assumed and not demonstrated. In the present paper we have obtained quantitative data from *in situ* experiments designed to demonstrate more definitively the effect of nutrient medium on muscle contractile capacity in adrenalectomized rats.

Methods. Male Rockland-Sherman rats weighing approximately 200 g were bilaterally adrenalectomized or sham operated under ether anesthesia and maintained postoperatively on tap water and Purina Chow *ad lib.* Under sodium pentobarbital anaesthesia, the triceps brachii muscle was freed distally at insertion with minimal damage to nervous and vascular elements and attached *in situ,* via the tendon, to a muscle lever so that contractions could be recorded with muscle immersed in oxygenated Krebs-Ringer phosphate medium (pH 7.4, 200 mg % added glucose, temp. 24 ± 2°C) contained in a chamber between muscle and recording lever. Muscle contractions were recorded by the Everse-DeFremery method(12) with some modifications. The isolated muscle was after-loaded with 10 g weight and under constant tension stimulated tetanically by maximal faradic stimuli through copper electrodes implanted in its proximal end. Each record was obtained by procedures of intermittent stimulation previously described(12). Three separate records were obtained at 25 minute intervals for each test muscle: the first representing initial contraction; the second corresponding to period of immersion in nutrient medium; and the third corresponding to period after removal from medium. Time intervals employed between records were those wherein maximal effects of immersion or removal of muscle from the medium were first observed. For descriptive and quantitative purposes, it has been assumed that, following initial fatigue, reduction in peak amplitudes of the last 3 contraction curves observed typically in each record (Fig. 1-4) is an index of recovery of muscle contractile capacity. By relating average peak height of these 3 curves to that of initial peak contraction, a relative value of recovery (expressed as percent) has been obtained for each record. Values thus obtained are inherently more uniform, since variations in contraction heights resulting from differences in muscle mass, initial tension, and other variables inherent in the recording method are invariably reflected in amplitude of initial curve and are thus internally compensated. Finally blood hematocrit values and body weights were obtained for all animals immediately after obtaining the third record.

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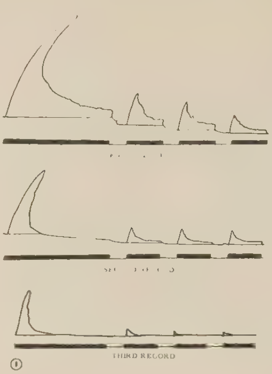
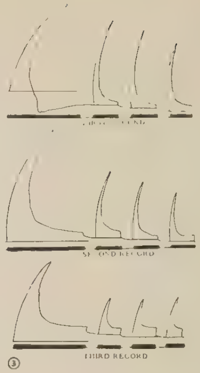
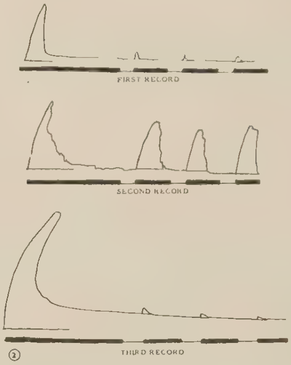
* Currently U. S. P. H. S. Pre-Doctoral Fellow in Pharmacology, Yale Univ. School of Medicine, New Haven, Conn.

† Present address: Dept. of Biology, Boston Univ., Boston, Mass.







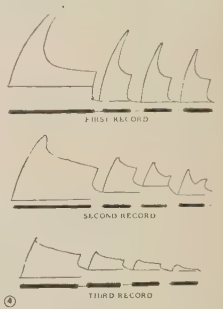


FIG. 1. Representative kymographic recordings of contractile capacity *in situ* of triceps brachii muscle in adrenalectomized rats obtained at 25 min. intervals without immersion of muscle in Krebs-Ringer medium.

FIG. 2. Same as Fig. 1 except that second record was obtained after immersion of test muscle for 25 min. in Krebs-Ringer medium and third record 25 min. after removal of bath.

FIG. 3. Representative kymographic recordings of contractile capacity *in situ* of triceps brachii muscle in sham operated rats obtained at 25 min. intervals without immersion of muscle in Krebs-Ringer medium.

FIG. 4. Same as Fig. 3 except that second record was obtained after immersion of test muscle for 25 min. in Krebs-Ringer medium and third record 25 min. after removal of the bath.

Results. Adrenalectomized Rats. Five rats bilaterally adrenalectomized 5 to 11 days previously and showing overt signs of moderate adrenal insufficiency were tested as described above without subjecting muscles to Krebs-Ringer medium. Fig. 1 shows representative records which demonstrate that recovery of muscle contraction is impaired following adrenalectomy. In 7 rats prepared as above, the muscle was immersed in Krebs-Ringer medium for 25 minutes immediately after completion of first record and prior to obtaining the second record. The medium was then removed, and the third record ob-

TABLE I. Recovery in Contractile Capacity of the *In Situ* Triceps Brachii Muscle Preparation of Intact and Adrenalectomized Male Albino Rats.

Procedure	Muscle treatment	No. of rats	No. of days postoperative	Mean change in body wt	Blood hematocrit	% recovery in muscle contraction		
						1st record	2nd record	3rd record
Sham operation	None	6	8	+35	40 ± 4*	63.8* ± 16.4	46.9 ± 7.7	43.2 ± 12.6
Idem	Krebs-Ringer	6	8	+27	44 ± 4	65.3 ± 18.7	46.0 ± 9.2	33.8 ± 9.4
Adrenalectomy	None	5	9	− 29	51 ± 4	17.9 ± 12.9	11.8 ± 10.8	4.5 ± 5.5
Idem	Krebs-Ringer	7	7	− 27	52 ± 3	19.2 ± 6.3	49.9 ± 10.1	3.9 ± 3.2
Adrenalectomy (no evident insufficiency)	None	2	9	+29	39	52.7	23.5	

* Mean ± S.D.

tained after 25 minutes. Fig. 2 shows that in adrenalectomized rats recovery of muscle contractile capacity is restored to normal following *in vitro* exposure of test muscle to Krebs-Ringer medium, but falls below pre-immersion levels upon subsequent removal of the medium.

Sham operated rats. Duplicate control experiments were performed with sham operated rats after postoperative recovery periods approximating those of the adrenalectomized group. Muscle contraction was recorded in 2 groups of 6 animals each. In one group muscles were not immersed in Krebs-Ringer medium; while in the second group, muscles were immersed prior to obtaining the second record. Time intervals employed between records in each case were the same as those described above. Fig. 3 shows that in absence of medium recovery of muscle contractile capacity is superior in sham operated rats to that observed in adrenalectomized rats under identical recording conditions (Fig. 1). Furthermore, exposure of the muscle to Krebs-Ringer medium for 25 minutes does not augment recovery of muscle contraction (Fig. 4).

Quantitative evaluation. Table I shows average muscle contraction recovery values for each of 3 records on all groups tested. From percent values of the second record, Krebs-Ringer medium had no apparent effect in sham operated rats. In contrast, adrenalectomized rats, compared with sham operated controls, showed an overall reduction of 40% (average of 3 records) in recovery of muscle contraction in absence of Krebs-Ringer medium with subsequent increase ($P<0.01$) in presence of medium to levels observed in second record of sham operated rats under similar conditions. Upon removal of the medium, recoveries declined to levels observed in third record of adrenalectomized untreated controls. Significantly, 2 adrenalectomized rats not showing overt signs of adrenal insufficiency were intermediate between sham operated rats and adrenalectomized rats with evidence of insufficiency. Reduction in muscle performance in complete insufficiency was accompanied characteristically by hemoconcentration and weight loss.

Discussion. Our demonstration that impaired muscle performance in adrenal insufficiency is restored to normal by immersion of isolated muscle in physiological medium strongly suggests, as responsible factors, impaired transport of ionic and/or metabolic factors or inadequate removal of toxic metabolites possibly as a consequence of impaired circulation. Wyman, Fulton, and Shulman (13) described extensive microcirculatory impairments in the cheek pouch of adrenalectomized hamsters. Further, it has been suggested(5,6) that impaired muscle performance in adrenal insufficiency may be, secondarily, a function of failure to make adequate neurocirculatory adjustments in time of physiological need. The effective role of circulation is further suggested by our observation that extensive chance circulatory damage to the triceps of sham operated rats prior to recording contraction invariably resulted in reduced performance which was restored by immersion of muscle in physiological medium. Alternatively, alterations in circulatory levels of ionic and/or metabolic factors necessary for efficient muscle contraction might be implicated. Hemoconcentration resulting from fluid loss implies ionic concentration and associated osmotic imbalance. Coupled with this are primary disturbances in serum electrolyte and carbohydrate levels. The latter do not appear to be important factors contributing to muscle fatigue of adrenal insufficiency, since it has been demonstrated that salt treated animals are incapable of sustained muscle activity despite normal glucose and serum electrolyte levels(14,15). In our experiments, however, hypoglycemia and serum electrolyte imbalance may be additional complicating factors, since adrenalectomized rats were not maintained on salt. Our limited preliminary observations on the role of metabolic factors indicate that omission of glucose and oxygen from Krebs-Ringer medium does not significantly impair its effect on increasing muscle performance in adrenal insufficiency.

Ingle(16) and Ingle, Hales, and Haslerud (17) demonstrated that, despite absence of overt signs of insufficiency, partial adrenalectomy reduces performance of muscle made to work over relatively long periods of time. Our present findings show that, presumably under similar conditions of insufficiency, muscle performance is likewise reduced although under relatively shorter periods of contraction.

Summary. Experiments were performed to evaluate quantitatively the effect of Krebs-Ringer medium on contractile capacity of muscle from adrenalectomized albino rats. The triceps brachii was used as test muscle and contraction recorded *in situ* using the method of Everse and DeFremery. Recovery in contractile capacity subsequent to initial fatiguing of test muscle was expressed as percent of initial contraction height. An overall reduction of 40% in recovery of contractile capacity was observed following adrenalectomy with accompanying hemoconcentration and body weight loss. Following immersion of test muscle in Krebs-Ringer-glucose-phosphate medium for 25 minutes, recovery was restored to levels observed in sham operated controls under similar conditions, but declined to control levels 25 minutes after removal of the nutrient bath. Possible underlying mechanisms are discussed.

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Quantitation of Monoamine Oxidase Inhibition Produced with Various Drugs in Man.* (25567)

Albert Sjoerdsma, John A. Oates and Louis Gillespie, Jr.
Section on Experimental Therapeutics, Nat. Heart Inst., Bethesda, Md.

Use of monoamine oxidase (MAO) inhibitors in treatment of such clinical conditions as psychic depression(1), hypertension(2,3), angina pectoris(4,5) and rheumatoid arthritis (6) constitutes a new and promising area of therapeutics. It seemed important to determine the enzyme-inhibiting potency of these newly-developed agents in man as well as to clarify the relationship between MAO inhibition and alleged therapeutic effects. To aid in accomplishing this, 2 methods were devised to quantify MAO inhibition in the human. The first consisted of measuring percentage of oral dose of serotonin excreted as urinary 5-hydroxyindoleacetic acid (5HIAA), before and after administration of a presumed MAO inhibitor(7). Secondly, we found that measurement of urinary excretion of tryptamine affords a convenient and sensitive method of estimating MAO inhibition in man(8). This report presents the effects of several MAO inhibitors of current clinical interest on excretion of tryptamine.

Methods. The subjects were 15 patients with uncomplicated hypertension. Twenty-four hour collections of urine were obtained during control and experimental periods and tryptamine was measured by method already reported(8). Five compounds designated as MAO inhibitors from animal studies were evaluated; these were iproniazid (Marsilid), B-phenylisopropylhydrazine (JB-516, Catron), *DL-phenylcyclopropylamine* (SKF-385), N-(2-(Benzylcarbamyl) ethylamino)-isonicotinamide (nialamide, Niamid) and phenelzine (Nardil). Dosage schedules included those suggested by the manufacturers and except for iproniazid the recommended daily dosage was exceeded to 2-fold in each instance to observe "maximal" enzymatic effects. The drugs were administered orally in single daily doses (iproniazid, JB-516) at beginning of each day's urine collection (8 a.m.) or initially in single daily doses and subsequently at 12, 8 or 6 hour intervals (SKF-385, nialamide and phenelzine). A specific dosage was maintained 5 to 14 days and changed only after daily excretion of tryptamine appeared constant. The tryptamine values for each dose-schedule were then recorded as the range occurring at time a new plateau had been reached.

Results. For purposes of comparison, alterations in urinary tryptamine and excretion of 5HIAA following oral administration of serotonin in a patient receiving JB-516 are shown in Fig. 1. A 70% inhibition of serotonin conversion to 5HIAA corresponded to a 5-fold increase in urinary tryptamine in this case.

Fig. 2 represents tryptamine excretion in another patient before and during administration of JB-516 and nialamide, the drugs given at separate periods of time. Daily excretion of tryptamine during administration of 25 mg/day JB-516 reached levels of 500-600 μg/day while a daily dose of 200 mg nialamide was required to produce a similar effect. Increasing the dosage of JB-516 to 50 mg/day failed to result in a proportionate increase of urinary tryptamine.

Table I is a summary of alterations in urinary tryptamine produced by various doses of the 5 inhibitors studied. A satisfactory dose-response relationship is apparent from the mean values of each group, particularly in the case of nialamide and SKF-385. However, it is also apparent that a rather wide range of values was obtained in different cases with a given dose of an inhibitor. A more reliable index of MAO inhibition for a given dose of drug results from expressing the ratio of average level of tryptamine excretion during experimental period to average level during con-

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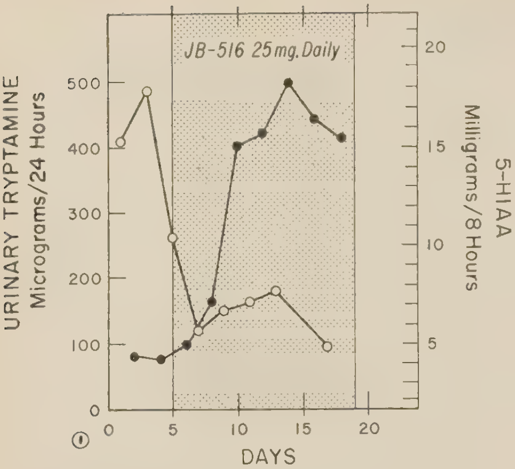
* We appreciate the technical assistance of Consuelo Muellenberg and thank the following organizations for supplies of drugs: iproniazid—Hoffmann-LaRoche; JB-516—Lakeside Labs.; SKF-385—Smith, Kline and French Labs.; nialamide—Chas. Pfizer and Co., and phenelzine—Warner-Chilcott.



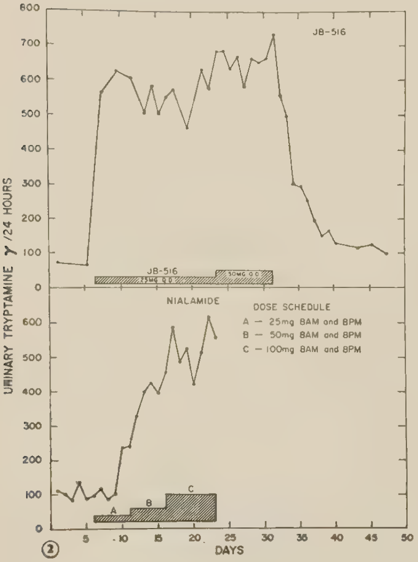


FIG. 1. Comparison of rise in tryptamine excretion (●) to fall in 5-HIAA excretion (○) during JB-516 administration.

FIG. 2. Urinary tryptamine excretion during sequential administration of JB-516 and nialamide in same patient.

trol period in each case individually. This obviates the variability of control levels of tryptamine excretion from patient to patient. An average of individual ratios at a given dose results in a mean ratio for the entire group. Range of individual ratios and mean ratios for each dose group is listed in last column in Table I.

A detailed account of other effects observed is beyond the scope of this paper. No overt psychic alterations were detected. A postural lowering of blood pressure was observed with 60-80 mg/day SKF-385, 120 mg/day phenelzine and in all cases to which JB-516 was administered. No alteration of blood pressure occurred with the reduced dose of iproniazid used or with nialamide at doses employed.

Discussion. These studies establish the drugs included in this report as MAO inhibitors in man. The daily doses of these agents, purported to give favorable results in psychiatric patients, are also the doses which result in at least a 4-fold increase in daily excretion of tryptamine (*e.g.* JB-516 - 12.5 mg; nialamide - 100-150 mg, and phenelzine - 60 mg). With the exception of nialamide, similar doses of the various drugs appear to produce a postural lowering of blood pressure in human hypertensives. It is important to note that nialamide, at doses used, failed to alter blood pressure even though tryptamine excretion was similar in amount to that observed with doses of other inhibitors which cause lowering of blood pressure. We have recently found, however, that higher doses of nialamide (400-500 mg/day) result in orthostatic lowering of blood pressure in hypertensives.

While increases in urinary tryptamine may not be exactly proportional to the effects of MAO inhibitors in every tissue, the method is simple and is nearest to a chemical test reflecting therapeutic activity now available. Also, conclusions can be drawn from studies on relatively few patients. The test appears to be quite sensitive, although it is not known to what extent MAO is actually inhibited in tissues at various levels of tryptamine excretion. It will be interesting to determine this on biopsy and autopsy material.

Other factors affecting use of urinary tryptamine as index of MAO inhibition have been discussed(8). One of these is the possibility of inhibition of tryptophan decarboxylation to tryptamine as might be expected to occur with certain hydrazine compounds; urinary tryptamine levels would thus fail to rise as much as with MAO inhibition alone. Also excretion of drug metabolites may interfere with the fluorimetric assay of tryptamine. In these respects the serotonin test may still be useful because of its specificity in indicating MAO

TABLE I. Effect of Various Doses of MAO Inhibitors on Urinary Tryptamine.

Drug	Daily dose (mg)	No. cases (No. determ.)	Urinary tryptamine (μg 24 hr)		Urinary tryptamine ratio,* avg during therapy/control avg	
			Range	Mean	Range (individual)	Mean (group)
Control		15 (59)	30– 111	63 ± 22†		
JB-516	12.5	2 (7)	115– 618	313	2.7– 7.0	5.3
	25	9 (54)	245– 750	484	5.2–10.0	7.2
	50	1 (9)	656– 825	704	11.3–14.2	12.1
Nialamide	50– 60	4 (15)	100– 234	157	1.7– 4.0	2.4
	100–120	5 (31)	209– 540	315	2.3– 7.8	4.3
	150	2 (8)	353– 515	444	6.2– 7.2	6.5
	200	4 (27)	337– 975	558	5.9–12.3	7.9
SKF-385	10	3 (9)	87– 159	120	1.5– 1.8	1.6
	20– 30	4 (14)	189– 544	281	2.2– 6.9	3.8
	40	2 (13)	255– 517	402	3.5–10.6	6.2
	60– 80	2 (21)	466–1030	719	5.6–13.0	10.6
Phenelzine	45	1 (5)	69– 100	85	1.3– 2.0	1.6
	60	2 (8)	120– 251	184	2.3– 6.1	4.0
	120	1 (3)	177– 512	299	4.3–12.5	7.3
Iproniazid	50	2 (15)	91– 231	161	1.0– 2.6	2.2

* See text for explanation. † One stand. dev.

inhibition and the fact that few agents interfere with assay of 5HIAA. Additionally, one may measure the increase in excretion of another amine, tyramine(9), where metabolism is also primarily dependent on MAO.

Summary. An increased urinary excretion of tryptamine occurred in human hypertensives during treatment with 5 monoamine oxidase inhibitors. Doses of drugs purported to produce comparable clinical effects, appear to have approximately equivalent enzymatic effects as indicated by changes in urinary tryptamine. It is recommended that biochemical observations of this type be included in further studies on therapeutic effects of these agents.

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Effects of Extracellular Potassium and Sodium on Intracellular Potassium and Sodium of Baker's Yeast.* (25568)

ADELAIDE BROKAW TOLBERG AND NELLO PACE
(With technical assistance of LaVerne Rohwer)
Dept. of Physiology, University of California, Berkeley

In studies of electrolyte exchanges in yeast, it was of interest to learn how the electrolyte content of yeast cells is affected by conditions of growth. Potassium is a major constituent of yeast ash, amounting to 23 to 40%(1). It is known that ash composition is affected by conditions of growth, but few data are available on effects of potassium and/or sodium content of growth medium. These experiments were undertaken to determine how the potassium content of yeast is affected by varying amounts of potassium chloride or sodium chloride in the growth medium.

Materials and methods. The yeast was a pure culture strain of *Saccharomyces cerevisiae* isolated from a cake of Fleischman's yeast. The basal growth medium contained 10 g/l Difco yeast extract and 50 g/l dextrose. This control medium contained approximately 8 meq/l each of potassium and sodium. Sterile salt solutions were added after autoclaving to produce final concentrations of approximately 30, 100 and 300 meq/l potassium or sodium. Each set of cultures consisted of one or, more frequently, 2 flasks of each cation concentration, so that comparisons could be made between cultures grown under same conditions. A uniform inoculum was used for any one set of flasks, and the cultures allowed to grow where temperature usually remained between 21 and 23°C, but might range from 20 to 25°C. Cells were harvested by centrifuging cultures 3 to 6 days after inoculation. It was assumed, from preliminary studies, that cultures had reached the stationary phase of growth at this time. The packed weight of centrifuged cells was used to estimate wet weight, assumed to be 78% of packed weight. This figure of Conway and Downey(2) was corroborated by us by estimating intercellular space (i) from packed weights of known amounts of cake yeast, and (ii) by measuring gelatin space of yeast harvested from laboratory cultures. Yeast was resuspended and an aliquot washed 3 or 4 times with about 10 ml water/g yeast for dry weight and electrolyte determinations. Because preliminary experiments indicated that some intracellular potassium was removed by washing procedure, electrolyte analyses were also made on unwashed aliquots. Corrections for extracellular potassium and sodium were made assuming extracellular solution accounted for the other 22% of packed weight of yeast (see above). Cations were extracted from yeast using Conway's procedure(3). Suspensions of both washed and unwashed cells (about 1 g/10 ml) were boiled 5 to 10 minutes. Supernatant cell extracts were appropriately diluted, as were samples of media, and potassium and sodium determined with a Perkin-Elmer Model 52C internal standard flame photometer. When compositions of cells grown on different media were compared, the data were paired with those of cultures from the same set; *i.e.*, grown at same time. The "t" test was used for calculation of P values.

Results are presented in Table I and summarized in Fig. 1.

Effects of varying potassium. When cultures from the same sets were compared, statistically significant differences could not be observed between Groups I and II. In Groups III and IV, however, there was significant reduction in water content of yeast with each increase in potassium concentration. Potassium content of unwashed cells increased with that of medium, especially at higher potassium concentrations. When yeast was washed, a considerable amount of potassium was removed from high-potassium cells so that Groups III and IV did not differ appreciably from one another, but still contained more potassium than Groups I and II. In all instances in which sodium content of cells was

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TABLE I. Water, Potassium and Sodium Content of Yeast Grown in Various Concentrations of Potassium or Sodium Chloride.*

	K or Na content of medium, meq/l	H_2O content of cells, g H_2O/kg wet cells	K content of cells Unwashed	K content of cells Washed†	Na content of cells Unwashed	Na content of cells Washed
			meq/kg wet cells			
A. Effects of varying potassium in medium; Na = 8 meq/l throughout.						
I.	8 ± .2‡	763 ± 7	101 ± 2	98 ± 3		
II.	29 ± .2	769 ± 6	109 ± 4	100 ± 4		
III.	107 ± 5	747 ± 9 (<.005)§	148 ± 8 (<.025)	119 ± 3 (<.05)		
IV.	303 ± 9	725 ±10 (<.001)	257 ± 9 (<.001)	120 ± 6		
B. Effects of varying sodium in medium; K = 8 meq/l throughout.						
V.	8 ± .4	767 ±10	112 ± 6	87 ± 4	8.4 ± .6	2.4 ± .6
VI.	34 ± 2	762 ± 8	106 ± 6	81 ± 3 (<.05)	19.9 ± .7	10.9 ± .5 (<.005)
VII.	139 ± 1	743 ± 9 (<.001)	97 ± 5	65 ± 1 (<.001)	78 ± 1	26.4 ± 1.9 (<.005)
VIII.	331 ±15	741 ± 7	104 ± 3	52 ± 5 (<.025)	194 ±17	37.4 ± 1.2 (<.05)

* Data derived from 4 to 6 sets of cultures except for those on sodium content of unwashed cells which were obtained from 2 sets.

† Washed cells were rinsed 3 or 4 times with approximately 10 ml water/g cells.

‡ Mean ± stand. error.

§ Figures in parentheses are P values for differences of results from those of preceding group.

determined, it was very low, usually less than 8 meq/kg wet cells, regardless of potassium content of medium.

Effects of varying sodium. Water content was reduced when sodium chloride concentration was increased above 100 meq/l. With 300 mM NaCl, however, there appeared to be no further decrease in water content. There was relatively little change in potassium content of cells grown in high sodium chloride, except for a slight decrease in Group VII. This difference was significant only in comparison with control Group V; P<.005. However, after washing, there was a marked decrease in potassium content with increasing sodium chloride concentration. With each increment in sodium chloride concentration in the medium, there was a rise in sodium content of both washed and unwashed cells.

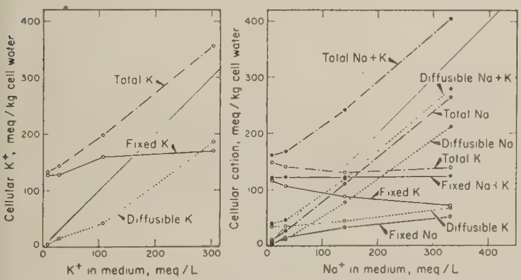

FIG. 1A (left). Effect of K content of growth medium on intracellular K of yeast cells.

FIG. 1B (right). Effect of Na content of growth medium on intracellular Na and K of yeast cells.

Fixed Na or K refers to that remaining after washing. Diffusible Na or K is calculated from difference between total ion and fixed ion concentrations. Straight line drawn at a 45° angle from the origin represents the curve which would be obtained if intra- and extracellular concentrations were equal.

Discussion. Total intracellular K concentration (meq/kg cell water) always increased with and exceeded extracellular K (Fig. 1A). The 2- to 3-fold rise in intracellular K suggests that yeast is poikilosmotic; *i.e.*, its internal osmotic pressure may vary over a wide range, depending on osmotic pressure of the external medium. This poikilosmotic character has been described in various lower organisms, including the bacterium, *Salmonella oranienburg*(4), and the marine alga, *Ulva lactuca*(5), both of which accumulate potassium in response to increased external osmotic pressure. Yeast differed from these organisms in that there was no increase in intracellular K when NaCl was substituted for the KCl supplements to the growth medium (Fig. 1B). However, the total intracellular cation concentration, as estimated from the sum of Na and K concentrations, gave a curve remarkably similar to that for K in Fig. 1A. In all cases intracellular Na concentration was lower than that of the medium, while intracellular K concentration exceeded it. The increase in intracellular cation concentration results not only from increase in total cation content, but also from decreased water content of yeast. Nonetheless, the water content was in no case as low as that of commercial yeast.

Yeast grown in high concentrations of either KCl or NaCl lost a considerable amount of potassium when washed with water. Christian(6) found a similar loss of K in washing experiments using *Salmonella oranienburg*. The loss from yeast was too large to be accounted for by possible inaccuracy in estimation of extracellular space, especially in the case of high Na where correction for extracellular K introduced almost no error. Similarly it cannot be accounted for by Conway and Downey's(2) "outer metabolic region" of the yeast cell. The efflux of K from baker's yeast described by Rothstein and Bruce(7) is probably not important in this case for several reasons: efflux rates are too small to account for losses in our experiments, particularly since each washing took 5 minutes or less; only negligible amounts of substrate were present; the efflux described by Rothstein and Bruce would be obliterated by the amounts of potassium in the wash water.

The present data suggest that yeast K exists in 2 fractions. One fraction (diffusible) is easily removed by washing and may account for almost half the intracellular K in cells grown in 300 mM KCl or NaCl. Since this fraction is relatively small (10 meq/kg cell water) in control cells, the increased diffusible K may be a consequence of increased osmotic pressure. The other fraction (fixed) is difficult to remove with washing. Intracellular Na may also be distributed into 2 compartments, although a smaller proportion appears to be in the fixed fraction (Fig. 1B). Fixed K does not change appreciably with increasing extracellular K, but decreases with higher extracellular Na concentrations. However, the sum of the fixed fractions of Na and K is constant over the range of Na concentrations studied, suggesting that Na competes with K for the fixed fraction.

Isotope studies have demonstrated that potassium is distributed in 2 or more fractions in several types of cells(8,9,10). The nature of the 2 fractions in yeast has not been ascertained. Possible factors include intracellular compartments of differing permeability or differential binding phenomena. Conway and Duggan(3) have shown a competitive effect of Na on K uptake by commercial yeast and have prepared Na-rich yeast in which Na has replaced intracellular K.

Summary. Experiments were carried out to elucidate the effects of varying concentrations of potassium and sodium in growth medium on water and respective ion content of yeast. The resulting changes in intracellular potassium and sodium indicate that yeast is a poikilosmotic organism; internal osmotic pressure as indicated by total intracellular sodium and potassium may vary 2- or 3-fold. Sodium and potassium appear to be distributed into at least 2 intracellular fractions; a diffusible fraction which is readily removed by washing, and a fixed fraction relatively constant in amount which is difficult to remove by washing. Sodium can replace over 40% of the fixed potassium at high external sodium concentrations.

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Variation in Epiphyseal Maturation of Medial Epicondyle of Humerus in Inbred Strains of Mice. (25569)

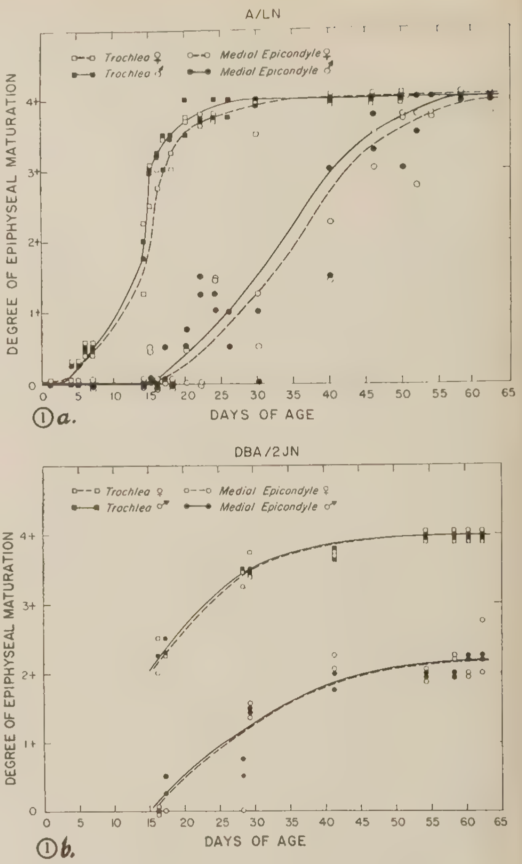
L. Sokoloff, G. E. Jay, Jr. and L. K. Randolph (Introduced by E. M. Lerner, II)
Lab. of Pathology and Histochemistry, Nat. Inst. of Arthritis and Metabolic Diseases and Nat. Cancer Inst., DHEW, Bethesda, Md.

In previous studies, patterns of development of epiphyses of knee and head of femur of mice during second year of life were described(1,2). These studies were carried out to determine sources of difference in occurrence of spontaneous degenerative joint disease in various inbred strains; and more particularly, whether development of osteoarthritis of the knee in various strains paralleled progression of epiphyseal closure as animals aged. No such relationship was observed. The present studies deal with ossification and closure of epiphyses during first 2 months of life rather than the second year. It was thus hoped to learn (1) whether there is a correspondence between rates of early and late epiphyseal development within individiual strains and (2) whether there is a relationship between early skeletal maturation, and later development of osteoarthritis.

Materials and methods. Two experiments were carried out: *Exp. A.* Development of epiphyses at distal end of right humerus was traced histologically in 2 groups of mice during the first 2 months of life as follows: Two male and 2 female littermates were killed at daily intervals during the first month and, non-littermates following weaning at intervals of 2 days until day 62. In the *first* group were strains A/HeN, A/LN, C3H/HeN and BALB/cAnN, born in February, and housed in groups of 4 or 5. All animals received Purina Lab. Chow and water *ad lib.* In the *second* group were C3H/HeN and DBA/2JN mice, born July and August, housed initially following weaning, in groups of 12 to 15 in large cages containing 2 food hoppers and water bottles, rather than shoebox type cages as in the previous group. As space became available, they were separated into smaller groups as in the preceding. Tissues were fixed either in neutral formalin or Bouin's solution. Decalcification and staining procedures were as previously described. Serial sections were made in a coronal plane. *Exp. B.* Specific ages of mice were chosen for comparing ossification of medial epicondyle with osteoarthritis of the knee, since some strains varied largely in these 2 measurements. The experiment included strains having earlier and later maturation times of the medial epicondyle and knee, as well as differences in occurrence of the joint disease (A/LN, DBA/2JN, STR/1N and C57BL/6JN). An attempt was made to obtain 15 pairs (littermates) of males for each strain. In each pair, 1 animal was killed at 60 days of age for examination of elbow, while the other was killed at 16 months for evaluation of arthritis. Intercurrent diseases reduced the number of animals in several instances (Table I). Production difficulties made consistent use of littermates impossible in STR/1N and precluded use of certain other strains, such as C57L/HeN which might have been desirable because they have a high degree of epiphyseal closure of the knee(1). Knees were macerated with papain and evaluated for osteoarthritis according to criteria described previously. The following scheme was employed for grading degree of epiphyseal maturation: 0 = no ossification of epiphysis; 4+ = complete ossification and union of epiphysis. It was possible to estimate the extent of ossification and of closure to the closest ½ in units of 1 to 4+. "Degree of epiphyseal maturation" was computed by dividing the sum of the grade of ossification and of closure by 2. It was noted, as observed by others, that foci of degenerated cartilage were often present in the epiphyseal plate without immediate vascular penetration or bony union. These were disregarded, and only vascular resorption of the plate or communication of hematopoietic tissue between diaphyseal and epiphyseal spaces recorded.

Description of epiphyses of distal humerus: Configuration and sequence of epiphyseal ossification of the distal humerus of mice corresponded closely to that described in the rat by Becks and coworkers(3). There was a main center of ossification in the trochlea that united with the diaphysis of the humerus immediately distal to the olecranon fossa, and a secondary center in the medial epicondyle. The plate of the latter was perpendicular to the former. The earliest changes in formation of the secondary center were a vesicular enlargement of lacunae of cartilage cells and penetration of blood vessels from the perichondrium into them. Epiphyseal plates were intact for a short time, focal disruption by penetrating blood vessels taking place before the epiphysis had completely expanded or ossified. The present method of grading degree of epiphyseal maturation has taken into account both degrees of ossification and of closure of the plate. The epiphyses of the distal end of the humerus of mice have been studied previously by Johnson(4) and Dawson(5). Secondary centers in the capitellum and trochlea were found by the former at 5 days, 11 hours of age; the medial condyle center at 9 days; and one in the lateral condyle at 19 days. Dawson observed the distal epiphysis to close at 21 days.

Results. Exp. A. In first and second groups, there was little variation between animals in rate of development of the distal epiphysis of the humerus within individual strains. Development was completed at 28 days of age in the A/LN mice; 32 days in BALB/cAnN; and at 36 days in A/HeN. Development of the medial epicondyle epiphysis was uniform in most strains (A/LN, C3H/HeN, BALB/cAnN; also DBA/2JN and STR/1N in Exp. B), but less so in 2 (A/HeN and, in experiment B, in C57BL/6JN. There was considerable variation between strains in degree of maturation achieved by the medial epicondyle by day 62. In strain A/LN, there was virtually complete ossification with fusion in all animals 52 or more days of age (Fig. 1A) while in others, as DBA/2JN (Fig. 1B), no fusion had taken place. The findings in the C3H/HeN animals were comparable regardless of whether animals were born in February or July. In neither trochlear nor medial epicondyle was an unequivocal difference in rate of maturation found between



①a. ①b.

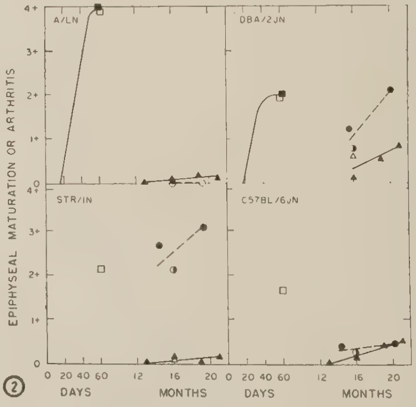


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FIG. 1. Rate of epiphyseal maturation at distal end of humerus in 2 inbred strains of mouse. *a.* A/LN; *b.* DBA/2JN.

FIG. 2. Comparison of development of epiphyses of medial epicondyle, knee and osteoarthritis in 4 strains of mice. ■ avg epiphyseal maturation of medial epicondyle (Exp. A); □ avg epiphyseal maturation of medial epicondyle (Exp. B); △ avg epiphyseal maturation of knee (concurrent exp., Ref. 9); ▲ avg epiphyseal maturation of knee (previous exp., Ref. 1); ○ (half-solid) avg osteoarthritis of knee (Exp. B); ● avg osteoarthritis of knee (previous exp., Ref. 1).

TABLE I. Maturation of Epiphysis of Medial Epicondyle and Osteoarthritis of Knees in 4 Strains of Mice (Males).

	Epiphyseal maturation*		Osteoarthritis of knees†	
Strain	No. animals	Avg degree (0-4+)	No. animals	Avg severity (0-4+)
A/LN	15	4.0 ± .2	14	0
DBA/2JN	15	2.0 ±0	15	.8 ±.18
STR/1N	14	2.1 ± .03	15	2.1 ±.31
C57BL/6JN	15	1.4 ± .33	11	.2 ±.26

* 60 days old. † 16 mo old.

the sexes; the process was more rapid in males, if anything.

Exp. B. Development of the epiphyses of the A/LN and DBA/2JN at 60 days was identical with those noted in Exp. A, and degree of osteoarthritis of the knees of the 4 strains quite comparable to that observed in the same strains several years earlier(2).

Our findings are summarized in Table I. The 2 strains having a low degree of osteoarthritis, A/LN (0) and C57BL/6JN (0.2), which are not significantly different with respect to this measurement ($t = .6$, $p \sim .5$), had, however, highly significantly different degrees of epiphyseal maturation (A/LN was 4.0; C57BL/6JN was only 1.4; $t = 7.67$, $p < .01$). The STR/1N and DBA/2JN mice had nearly identical degrees of epiphyseal maturation (2.1 and 2.0 respectively) but differences in severity of osteoarthritis (2.1 and 0.8) were highly significant ($t = 3.58$, $p < .01$) although there was wide variation within a strain.

Discussion. Our studies demonstrate that early closing epiphysis of the medial epicondyle of the humerus develops at different rates in various strains of mice. These rates are quite constant in these strains over many generations. They were not affected, within limits of experiments, by season in which animals were born or numbers housed together. Although infantile diarrhea occurred in the C3H/HeN colony in Exp. A, first group, the mice studied here were selected to be unaffected by it and had bone changes comparable to those in Exp. A, second group, where they had normal weight curves.

No distinct sex differences in rate of epiphyseal maturation of the elbow were observed. This is in contrast to findings in late closing epiphyses of hip and knee(1,2) in which ossification is more advanced in males. The findings are at some variance with the observations of Silberberg and Silberberg(6), that during early development the female mouse skeleton is physiologically older than the male, although the male thereafter may overtake and exceed the female. Comparison of humeral maturation with that of the knee of the same strain indicates that there is no consistent relationship between rate at which epiphyses mature early in life in mice and ultimate progression of those that remain open till late age (Fig. 2).

Silberberg and Silberberg observed that the rate at which epiphyseal cartilage undergoes the sequence of proliferation, regression and bony union, *i.e.,* skeletal time curve, varies among different strains of mice. C57BL mice, showing slow skeletal growth, had more spontaneous degenerative joint disease and responded to high fat diet with accelerated skeletal aging and more osteoarthritis than did DBA mice that had a rapid skeletal growth under ordinary conditions(7). Furthermore, young mice were more susceptible to accelerated epiphyseal and articular cartilage aging, induced by high-fat intake for 5 months, than were older ones(8). Because previous studies(1,2) failed to disclose a close relationship between late epiphyseal aging and articular degeneration in mice, we inquired whether the differences in prevalence of spontaneous osteoarthritis in different strains of mice correspond to early changes in skeletal development rather than late ones. We demonstrated that maturation of early closing medial epicondyle epiphysis does not bear any relationship to ultimate development of osteoarthritis in the same strain. Thus, sources of differences in which spontaneous osteoarthritis occur with varying frequency in different strains of mice must be sought outside early or late rates of skeletal "aging" of these strains.

Summary and conclusions. Rate of ossification and closure of epiphyses of the medial epicondyle of the humerus was studied in 7 inbred strains of mice. The rate of development varied significantly in different strains but bore no relationship to occurrence of degenerative joint disease, or to ultimate epiphyseal development of knee or head of femur previously found to occur late in second year of life.

Grateul acknowledgement for statistical computations is made to Joan Gurian.

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Heterogeneity of Insulin-Antibody Complexes in Rabbits and Guinea Pigs.

(25570)

JANE H. MORSE* (Introduced by S. A. Berson)
Vet. Admin. Hosp., Bronx, N. Y. City

Presence of insulin-binding antibodies in human subjects treated with commercial mixtures of beef, pork insulin(1) and in guinea pigs and rabbits immunized with beef or pork insulin(2) has been established with use of insulin-I^{131} and paper chromatoelectrophoresis of insulin antiserum mixtures. Kinetic studies of rates of formation and dissociation of insulin-antibody complexes and analysis of equilibrium mixtures have indicated the heterogeneity of complexes formed in human antiserums(3-6). Heterogeneity in this case appears to be due to presence of 2 (or more) distinct orders of antibody combining-sites rather than to differences in stoichiometric proportions of insulin and antibody in the complexes(5,6). Since human antiserums contain antibodies to both beef and pork insulins, heterogeneity of insulin-antibody complexes might be due to antibodies directed against different antigenic determinants in beef and pork insulin or to separate antibodies directed against multiple distinct antigenic sites in each insulin derived from a single species(6). In our study guinea pigs and rabbits have been immunized with *either* beef *or* pork insulin. Insulin-binding antibodies in antiserums of these animals react with insulin to form heterogeneous antigen-antibody complexes.

Methods. Analytic and technical methods follow procedures described by Berson and Yalow(1,5,6). In "equilibrium state" studies, mixtures of antiserum and beef or pork insulin (containing trace amounts of same species' insulin-I^{131}) were incubated at 37° until equilibrium was reached (usually about 4 hours) and then analyzed by paper chromatoelectrophoresis for insulin bound to antibody(B) and unbound ("free") insulin (F). Considerations based on the law of mass action indicate that when antiserum concentration is kept constant while insulin concentration is varied, the ratio B/F is a linear function of concentration of bound insulin (B) only *if* insulin is univalent *and* all antibody combining-sites are of a single order(5,6). A curvilinear relationship is expected if insulin is multivalent and/or if there is more than a single order of antibody combining-sites. In 5 guinea pig and 3 rabbit antiserums studied, plots of B/F *vs.* B were curvilinear (Fig. 1), a finding which is consistent with either of the last 2 alternatives.

Results. In "transient state" studies, only dissociation experiments are analyzed. Following attainment of equilibrium between bound and free insulin, high concentrations of unlabeled insulin were added to the mixtures. Further binding of insulin-I^{131} is now competitively inhibited and rate of "dissociation" of insulin antibody complexes is determined from decrease in concentration of bound insulin-I^{131} with time. The complexes exhibited heterogeneity in rates of dissociation in all experiments. In the 2 rabbit antiserums analyzed, the dissociation curves could be resolved into 2 components with half times averaging 9 and 8 minutes and 2¼ and 7 hours respectively (Fig. 2a). However the 5 guinea pig antiserums appeared to have a greater degree of heterogeneity, and resolution of the experimental curves required more than 2 exponential components (Fig. 2b).

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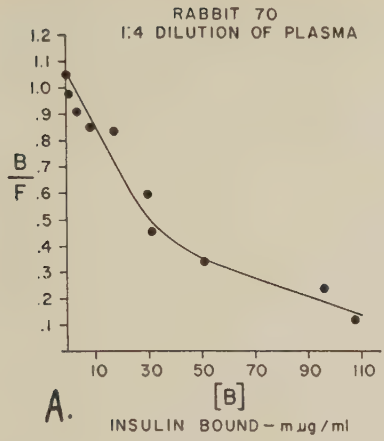
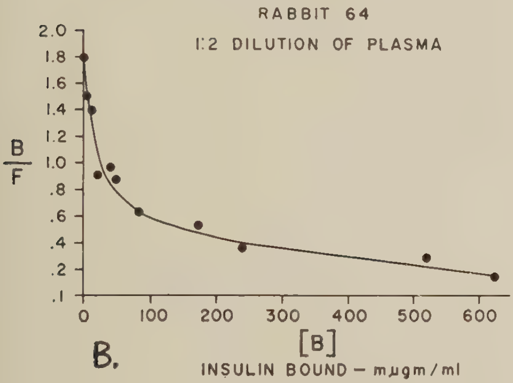
* Fellow Nat. Fn.

Discussion. That antigen-antibody com-





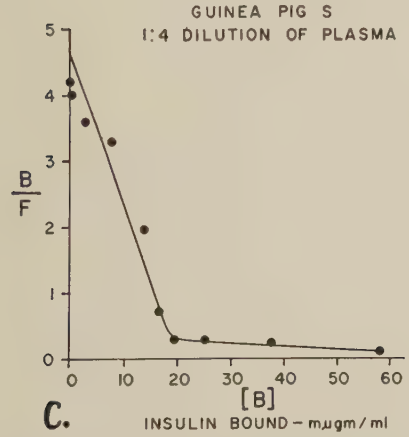
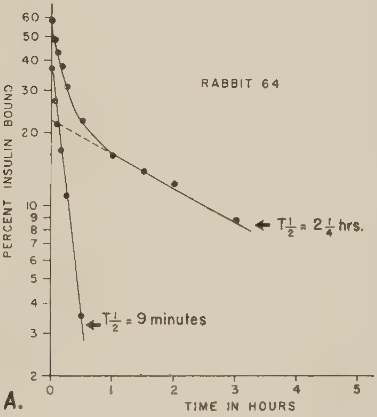


FIG. 1A. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-beef insulin plasma of a rabbit incubated with crystalline beef insulin.

FIG. 1B. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-pork insulin plasma of a rabbit incubated with crystalline pork insulin.

FIG. 1C. Ratio of bound to free insulin as a function of quantity of insulin bound for anti-beef insulin plasma of a guinea pig incubated with crystalline beef insulin.



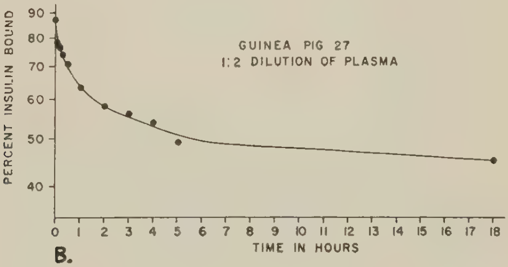


FIG. 2A. A typical rate curve of dissociation for rabbit insulin-antibody complexes. This plasma was from a rabbit immunized with pork insulin and was incubated with crystalline pork insulin-I^{131}.

FIG. 2B. A typical rate curve of dissociation for guinea pig insulin-antibody complexes. This plasma was from a guinea pig immunized with beef insulin and was incubated with beef insulin-I^{131}.

plexes are heterogeneous† is generally not unexpected inasmuch as numerous sites of antigenic determinancy on most antigen molecules are responsible for multivalency of antigen in its reaction with antibody. With insulin as an antigen, however, univalency might be anticipated from studies of Harris, Sanger and Naughton(7). These authors showed that insulins from 5 different mammalian species (cattle, sheep, pig, horse, whale) differ in amino acid sequence only in the 8-10 positions of A chain. Immunologic differences in reaction of these insulins with human anti-beef, pork insulin serums suggest that this region includes a site antigenic for man(8,11). If guinea pig and rabbit insulin likewise differ from other mammalian insulins only in this respect it might be expected that antigenicity would reside only in the relatively small site in question and that the insulin monomer (M. W. 6000) could therefore bind only a single antibody molecule under any circumstance. Moreover, even if beef or pork insulin exhibits more than a single antigenic site to guinea pigs and rabbits, the small size of the insulin molecule might still preclude the binding of 2 (or more) antibody molecules simply because of steric hindrance(11). The possibility that beef or pork insulin possesses other sites of possible antigenicity in guinea pigs and rabbits cannot be excluded until the structures of guinea pig and rabbit insulin are established. (Lacy and Davies(12) obtained evidence, with fluoroscein labeled insulin antibodies, that guinea pig and rabbit insulins are immunologically different from beef insulin and several other mammalian insulins.)

From data presented here it can only be concluded that *either* insulin is multivalent *or* that the antibodies are heterogeneous or, lastly, that both conditions exist. In human antiserums the weight of evidence(6) appears to favor univalency (though not necessarily monoantigenicity). A more complete description of the nature of insulin-antibody complexes in rabbits and guinea pigs must await more extensive studies with these antiserums.

Summary and conclusions. Heterogeneity of insulin-antibody complexes in antiserums of rabbits and guinea pigs immunized with *either* beef insulin *or* pork insulin has been demonstrated. Heterogeneity of complexes in antiserums of human subjects immunized with commercial mixtures of beef *and* pork insulins need not be attributed to presence of distinct antibodies directed against beef and pork insulins.

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† Although heterogeneity of complexes is observed even with univalent haptens(9,10), the heterogeneity is related to Gaussian distribution of binding energies and is rather less marked than in insulin-antibody complexes which, in man(5,6) and rabbits exhibit 2 main groups with grossly different dissociation rates.

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Preparation and Use of I^{131} Labeled Sodium Iodohippurate in Kidney Function Tests. (25571)

MANUEL TUBIS, EDWARD POSNICK AND ROBERT A. NORDYKE
(Introduced by A. L. Sellers)
Radioisotope Service, V. A. Center, Los Angeles, Calif. and Depts. of Radiology and Medicine, School of Medicine, University of Calif., Los Angeles

There is need for a rapid and non-traumatic method for assessing individual kidney function. Use of I^{131} labeled compounds and external radioactivity measurements over the kidneys appear quite adequate for the purpose. After injection, uptake and clearance by each kidney are measured by means of radioactivity, producing a printed record by use of appropriate instrumentation(1). However, the method used previously was complicated because the diethanolamine salt of I^{131} labeled 3,5-diiodo-4-pyridone-N-acetic acid (Diodrast) was not kidney-specific and because of its hepatic uptake, required careful placement of the probes(2). In addition, I^{131} labeled analogs of sodium 3,5-diacetamido-2,4,6-triiodobenzoate (Hypaque), methyl-glucamine 3,5-diacetamido-2,4,6-triiodobenzoate (Renografin), sodium 3-acetamido-2,4,6-triiodobenzoate (Urokon), and sodium 3,5-dipropionylamino-2,4,6-triiodobenzoate (Miokon), all have the disadvantage of being cleared slowly by the kidneys, prolonging test time to 15-25 minutes. Although they are essentially not picked up by the liver, they differentiate less clearly the function of the individual kidneys. Theoretical considerations indicated that sodium o-iodohippurate (Hippuran) had the desired characteristics for comparing separate kidney function, namely rapid and complete removal from the blood by the kidneys only. This compound containing I^{131} was then prepared by the 2 methods described and proved quite satisfactory for the purpose.

Methods. Hippuran containing I^{131} was prepared by 2 methods of exchange. In *Method I,* ca. 20 mc I^{131} was released from NaI^{131} solution (Oak Ridge) by 0.33 ml 0.01 M KI, 0.1 ml M $NaNO_2$ and 0.2 ml 2.5 M HCl and the iodine shaken out with 2 ml CCl_4. The shakeout was repeated with the same quantities of reagents but no I^{131}. The combined CCl_4 solutions of I^{131} were washed by shaking with 2 ml H_2O, then extracted with 0.5 ml H_2O plus 0.6 ml 0.1 M NaOH followed by 0.5 ml H_2O plus 0.2 ml 0.02 M NaOH. The NaI^{131} solutions were added to 363 mg Hippuran dissolved in 2 ml H_2O in 10 ml serum vial and adjusted to pH 6 with 0.1 M HCl and pH paper. The vial was closed with rubber stopper covered on underside with Saran film, sealed with aluminum seal and heated 2 hours in boiling water. The contents were transferred to 20 ml beaker and evaporated to dryness, redissolved in 1 ml boiling H_2O and transferred to 12 ml centrifuge tube. The o-iodohippuric acid was then precipitated with 0.6 ml (1 + 9) HCl, centrifuged 2500 rpm/10 min., the supernate removed, the precipitate dissolved in ca. 4.5 ml boiling H_2O, 1 mg KI in 0.1 ml H_2O added and solution cooled in ice water and centrifuged. The precipitate was transferred using a minimum amount of supernate to a small sintered glass filter, washed with small portions of ice-cold saturated solution of non-radioactive o-iodohippuric acid and dried to constant weight at 100°. The yield was 55% of the weight of Hippuran taken and specific activity 49 μc/mg. The m.p. was 173.5° (corr.), which differed from that reported by Novello(3) but agreed with another source which reported 171-174°(4). In *Method II,* I^{131} was released by same quantities of reagents as in Method I into 2 ml chloroform. The chloroform extracts were water-washed and added to 363 mg Hippuran and 10 μg ICl in 0.1 ml $CHCl_3$, in 5 ml absolute alcohol in 30 ml ground glass neck flask equipped with reflux condenser. The mixture was refluxed for 8-9 hours, the condenser removed and evaporated to dryness. The residue was dissolved in 1 ml boiling H_2O, transferred to 12 ml centrifuge tube, the acid released and purified by recrystallization as in Method I.

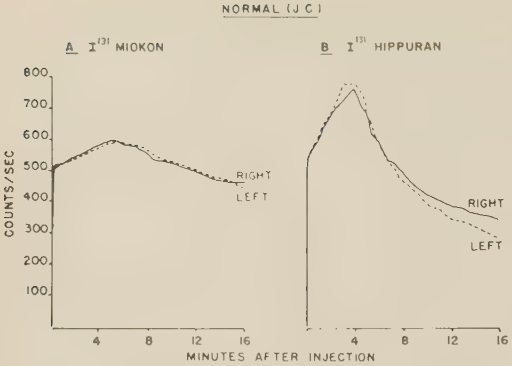

FIG. 1. Comparison of clearance of I^{131} labeled Miokon and I^{131} labeled Hippuran by normal human kidneys.

When dried to constant weight, had same properties and had a specific activity of 65 μc/mg, and same melting point. Solutions containing 25 μc/0.75 ml for parenteral use were made by dissolving requisite quantity of the o-radioiodohippuric acid in stoichiometric amount of 10% Na_2CO_3 solution, diluting with sterile pyrogen-free H_2O, filtering through sintered glass and sterilizing in stoppered and sealed serum vials in boiling water for 30 minutes.

Results. Radioiodinated Hippuran of high purity and adequate specific activity has been prepared by 2 methods. After doses of ca. 25 μc were injected into normal individuals and patients with suspected renal pathology, radioactivity over each kidney area was recorded by 2 matched scintillation probes, ratemeters, and a dual rectilinear recorder. A typical pattern for a normal human, using I^{131} labeled Hippuran, is shown in Fig. 1B, which demonstrates individual kidney function. The more rapid clearance of I^{131} Hippuran compared with I^{131} labeled Miokon is shown in Fig. 1.

The curves are almost identical for right and left kidneys, indicating no uptake by the liver, which would have affected the right kidney reading (Fig. 1B). Additional confirmation was provided by the virtual absence of radioactivity in bile obtained from a common duct T-tube in a patient with normal kidney and liver functions. The lack of liver uptake permitted placement of probes perpendicularly to patient's back, directly over the kidneys, greatly simplifying collimation. Since I^{131} labeled Hippuran is removed by the kidney more rapidly and completely than I^{131} labeled Miokon, Hypaque and Renografin, testing time was reduced to less than 10 minutes. The rapid extraction magnified the absolute function and differences between the 2 kidneys.

Summary. I^{131} labeled sodium o-iodohippurate (I^{131} Hippuran) has been prepared by 2 methods. This compound eliminates major disadvantages of currently used radioactive substances for individual kidney function testing. There is no complication due to liver uptake. It is more rapidly and completely removed, thereby reducing the time for test by 100% or more. Clinical studies with patients having known urinary tract pathology indicate that I^{131} Hippuran demonstrated comparative differences more sensitively than other labeled "renographic" substances(5).

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Effect of Insulin on Subcutaneous Absorption of Drugs in Rats.* (25572)

GAETAN JASMIN,† AND ANDRE BONIN
Dépt. d'Anatomie Pathologique, Université de Montréal, Montreal, Canada

Previous studies have shown that insulin, in minimal doses, increases rat's sensitivity to dextran edema(1). It was assumed that insulin activates the reaction by other means than hypoglycemia, possibly by enhancing penetration of dextran into the tissues. This led us to investigate the effect of insulin on urethane anesthesia and on convulsions produced by dimethyl-p-phenylenediamine(2). The influence of insulin on subcutaneous absorption was also assessed by intravenous administration of Evans blue in animals already injected intradermally with physiologic saline.

Materials and methods. Seventy-two female rats (Holtzman) weighing 140 to 160 g were fasted 6 hours before absorption tests. In each experiment, animals were divided into groups of 12 and received 0.2 I.U. of zinc-insulin (Connaught), subcutaneously, 15 minutes before injection of other substances. Ethyl urethane or dimethyl-p-phenylenediamine was given subcutaneously at 150 mg and 4 mg, respectively, each in 0.2 ml of saline. Degree of anesthesia and convulsions was appraised visually according to arbitrary scale of 0 to 4+. Readings were made at 10, 20 and 30 minutes after injection of drugs and our results are expressed as percentages of maximal possible response (4+). For salt solution absorption test, 0.2 ml of physiologic saline was injected intradermally into 3 different regions of dorsal skin. This was followed by intravenous injection of 0.2 ml of 0.5% solution of Evans blue. Ten minutes later, animals were sacrificed and dorsal skin was dissected from underlying muscle and turned over for appraisal of intensity of blueing at site of injection of physiologic saline (Fig. 1). Readings were made according to relative scale of 0 to 4+. Since blueing was inversely proportional to rate of absorption, our percentual values (Table I) were calculated as follows: 0 = 100; + = 75; 2+ = 50; 3+ = 25; 4+ = 0. A few minutes before animals were killed, 1 ml of blood was taken from jugular vein for sugar determinations made according to method of Folin Wu.

Results. Mean values (Table I) reveal that activity of urethane was definitely increased by insulin. The promoting effect on anesthesia was readily demonstrable during 30 minutes following injection of relatively small doses of urethane. After this period, the differences were less evident, apart from slow recovery of insulin-treated rats. In dimethyl-p-phenylenediamine, the effect of insulin was more clear cut: latent period of convulsions was reduced by 10 minutes. Although blood sugar values were significantly lowered by insulin treatment, they remained within the lower limits of normality.

Our observations from intradermal saline tests are in line with above findings. The percentage of absorption, as judged by low degree of blueing reaction, was markedly increased in insulin-treated rats (Fig. 1).

Discussion. These results support the concept that insulin enhances absorption rate of substances injected subcutaneously. Thus, it seems reasonable to assume that the promot-

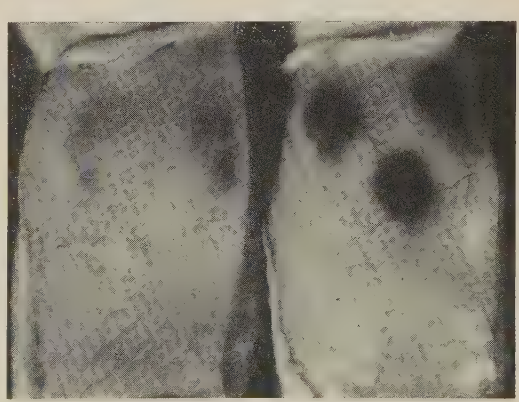

FIG. 1. Effect of insulin on subcut. absorption of physiologic saline. Relative blueing reaction at sites of subcut. inj. of saline. *Left*, insulin-treated rat. *Right*, control.

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† Medical Research Associate of Nat. Research Council of Canada.

TABLE I. Effect of Insulin on Induction of Urethane Anesthesia, Dimethyl-p-Phenylenediamine Convulsions and Subcutaneous Absorption of Physiologic Saline in Rats.

Groups of 12 rats	Urethane* anesthesia (%) 10 min.	20 min.	30 min.	Glycemia,† mg %	PPD‡ convulsions (%) 10 min.	20 min.	30 min.	Glycemia,† mg %
Controls	0	13	25	112 ± 3.1	0	0	90	128 ± 4.2
Zinc-insulin (0.2 I.U. s.c.)	25	37	67	78 ± 5.9	0	90	100	80 ± 5.3

	Absorption rate of saline (%)§	Glycemia,† mg %
Controls	39	118 ± 3.7
Zinc-insulin (0.2 I.U. s.c.)	80	74 ± 6.1

* Mean readings following s.c. inj. of 100 mg/100 g of ethyl urethane.
† Mean ± S.E.
‡ Mean readings following s.c. inj. of 2.6 mg/100 g of dimethyl-p-phenylenediamine.
§ Mean percentage based on intensity of blueing 10 min. after i.v. inj. of 0.2 ml of 0.5% Evans blue.

ing effect of insulin on dextran reaction is also derived from increase in absorption. Whether insulin influences the reaction by changing permeability of cells or of ground substance remains to be shown. It has been reported that subcutaneous absorption of urethane is augmented by cortisone, in rats, and by dehydration and fasting, in mice. This was interpreted as resulting from a reduction of hyaluronic acid content in connective tissue ground substance(3). Insulin may act similarly, perhaps by influencing activity of hyaluronidase.

Fasting, especially in adrenalectomized rats, augments sensitivity to dextran(1), and similarly, activity of various drugs was more uniform in animals subjected to a short period of fasting. It is also common knowledge that the efficiency of therapeutic agents, even when given parenterally, is higher in fasting patients. That a slight decrease in glycemia, within physiologic range, activates absorption phenomena may also explain the fact that insulin given at a low dosage promotes absorption of substances injected subcutaneously.

Summary. Subcutaneous absorption of urethane and of dimethyl-p-phenylenediamine was augmented by insulin, as evidenced by acceleration of anesthesia and convulsions caused by these substances, respectively. Insulin also promoted absorption rate of intradermally injected physiologic saline.

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Potassium Metabolism in Rats with Chronic Renal Insufficiency.* (25573)

ASHTON B. MORRISON (Introduced by J. Russell Elkinton)
Dept. of Pathology, School of Medicine, University of Pennsylvania, Philadelphia

Male rats which had been subtotally (5/6) nephrectomized developed a form of chronic renal insufficiency similar in many ways to that occurring in the human patient with chronic nephritis. At death, lesions were found in the cardiovascular system and in bones of the rats, which were indistinguishable in quality from those found in some patients with chronic renal insufficiency(1). When at least one month had elapsed following operation the adrenals were enlarged(1). This enlargement in such animals suggested that during period of renal insufficiency the adrenals

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TABLE I. Effect of Low Potassium Diet on Muscle and Serum Potassium, Serum CO_2 Content and Myocardium of Subtotally Nephrectomized and Sham Control Rats.

Group	No. of rats	Treatment		Serum		Muscle K as meq/100 g f.f.s.	Myocardial lesions grading
		Operation	Diet	Total CO_2, mM/l	Potassium, meq/l		
A	8	Subtotal nephrectomy	Low K	32.4 ± 1.6[*]	2.9 ± .2	35.4 ± 1.8	2+ to 4+
B	8	*Idem*	Control	24.6 ± .7	4.6 ± .3	48.1 ± 1.5	0 to 1+
C	8	Sham operation	Low K	32.2 ± .9	3.8 ± .2	41.5 ± 1.3	0 to 2+
D	8	*Idem*	Control	26.1 ± 1.1	4.6 ± .1	47.1 ± 1.2	0 to 1+
				p[†]	A & C < .01	A & C < .05	

f.f.s. = fat-free solids.

[*] Means ± stand. errors. Stand. errors calculated as $\sqrt{\Sigma (\mathrm{x} - x)^2 \Big/ \frac{n-1}{n}} = \sigma$. x, mean of x.

[†] p determined from ± test of differences, D, between means of 2 groups where $\sigma D = \sqrt{\sigma x_1^2 + \sigma \mathrm{x}_2^2}$, $t = \frac{D}{\sigma D}$.

were more active than in the normal animal. Adrenal overactivity may lead to potassium depletion(2), and if adrenals of subtotally (5/6) nephrectomized rats were overactive, it might be expected that such animals would show a tendency to become potassium depleted more readily than normal rats. To test this, it was decided to feed subtotally (5/6) nephrectomized rats a low potassium diet and discover whether they became more potassium depleted than sham operated control rats fed the same diet. The criteria used to assess potassium depletion in the rats were, fall in serum potassium levels, rise in total CO_2 of serum (resulting from metabolic alkalosis of potassium depletion), extent of depletion of muscle potassium, and degree of focal myocardial necrosis.

Materials and methods. Male Wistar rats weighing 250 g were subtotally (5/6) nephrectomized. Sham operations were carried out on another group. Six weeks after operation, half of the sham control group (Group C) and half of subtotally nephrectomized group (Group A) were fed a diet low in potassium[†] but containing 30 meq of magnesium/kg of diet and adequate in all other nutrients. Remaining animals (Groups B and D) were fed the same diet to which potassium had been added (0.85 g KH_2PO_4 and 0.58 g KCl to 100 g diet). This diet was continued 70 days, when blood was taken under oil from the aorta in each animal and the animal killed. The muscle was analyzed for potassium by the method of Darrow(3) using a Baird flame photometer. Serum CO_2 content was measured manometrically by the method of Van Slyke and Neill(4). The heart was fixed, bisected and after embedding was sectioned so that the myocardium of each ventricle and auricle might be examined. The extent of myocardial lesion was graded 0, 1+, 2+, 3+, or 4+ depending on number of lesions seen on low-power examination of a couple of complete sections through middle of heart, by an observer who had no knowledge of the grouping of the animals.

Results are presented in Table I. There was clearly a greater depletion of muscle potassium in subtotally (5/6) nephrectomized animals (Group A) than in sham operated controls (Group C), when both were on a low potassium diet. Serum potassium levels were also significantly lower in the subtotally nephrectomized group, than in the sham operated control group on a low potassium diet. In studies by others(5), degree of elevation of serum CO_2 in potassium deficient rats is correlated closely with the fall in muscle potassium, but in animals reported here, where there was clearly a greater degree of muscle potassium depletion in sub-totally nephrectomized animals (Group A) than in sham operated controls (Group C), there was no significant difference in serum CO_2 content. The most severe myocardial focal necrosis and fibrosis was observed in subtotally nephrectomized animals made potassium deficient (Group A). These lesions were very much more severe in subtotally nephrectomized potassium depleted animals than in sham animals subjected to potassium deficiency. Degree of necrosis and scarring naturally varied from animal to animal, but in none of the sham controls of Group C was the lesion as severe as in the least affected of subtotally nephrectomized group (Group A).

[†] Low Potassium Diet bought from Nutritional Biochemicals Corp., Cleveland. Composition: cornstarch, 64%; casein, 30%; butterfat, 3.5%; calcium carbonate, 1.3%; sodium chloride, 1%; and an adequate supplement of all known vitamins. The diet on analysis contained 8 meq potassium/kg, 340 meq sodium/kg, and 7.0 meq magnesium/kg. A mixture of magnesium chloride and magnesium sulphate was added to bring level of magnesium in stock diet to 30 meq of magnesium/kg of diet.

Discussion. These findings indicate that rats with chronic renal insufficiency induced by subtotal (5/6) nephrectomy become more potassium deficient on a low potassium diet than sham-operated control animals on the same diet. While this finding is consistent with adrenal cortical overactivity in subtotally nephrectomized rats, there remains the possibility that the increased loss of potassium resulted directly from impaired renal function. When such sub-totally nephrectomized animals are on a normal diet, excretion of potassium is no greater after than before subtotal nephrectomy(6). Preliminary experiments have shown that a sub-totally nephrectomized rat can reduce its urinary loss of potassium in much the same fashion as a normal rat, when placed on a low potassium intake. These facts suggest that nothing like a form of potassium losing nephropathy is induced by the operation. Whatever may be the initiating factor in the overall increased loss of potassium, when subtotally nephrectomized animals are placed on a low potassium diet, it seems likely that increased secretion of adrenocortical hormones is involved in the process. Increased secretion of adrenocortical hormones might also explain the greater incidence and severity of myocardial lesions in rats with a subtotal nephrectomy on a low potassium diet (Group A), than in sham controls on the same diet (Group C), since administration of adrenocortical hormones increases susceptibility of the myocardium to damage in potassium deficiency(7,8). In previous experiments(9,10) subtotally (5/6) nephrectomized rats formed and excreted more urea and lost more potassium than sham operated controls, when both were starved for 4 days. Such findings were again possibly the result of greater adrenal activity in subtotally (5/6) nephrectomized animals. These observations concerning potassium metabolism of rats with chronic renal insufficiency may have a bearing on metabolic derangements in chronic nephritis in the human, and are to be reported elsewhere in detail.

Summary. Subtotally 5/6 nephrectomized rats and sham operated controls were fed a diet deficient in potassium. Subtotally nephrectomized animals had significantly less potassium in their muscles, and a lower serum potassium than control animals; there appeared to be no difference in serum CO_2 content in the 2 groups; subtotally nephrectomized rats showed more focal necrosis and fibrosis of the myocardium than control animals on a low potassium diet.

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Phosphorus Metabolism in Infection with Murine Leukemia Virus. (25574)

HILTON B. LEVY AND LEROY F. SNELLBAKER
U. S. Dept. of H.E.W., P.H.S., Nat. Inst. of Allergy and Infect. Dis., Lab. of Biology of Viruses, Bethesda, Md.

Our previous reports(1,2) indicated that there is increased *in vivo* uptake of P^{32} by spleens of mice infected with leukemia-inducing virus described by Friend(3), as compared with non-infected controls. This increased uptake of P^{32} occurs before the infected spleen shows enlargement characteristic of the disease. At this early stage of infection little or no pathological alterations can be seen, and little or no new virus has been formed. Under conditions in these tests stimulation of P^{32} uptake is to some degree specifically related to this disease, in that it is not seen in some other diseases characterized by spleen enlargement or pathological changes. These other diseases are histoplasmosis, lymphocytic choriomeningitis, and several transplanted leukemias. On the other hand, spontaneous leukemia in AKR mice and a leukemia induced by Gross's virus (4) do show the stimulated P^{32} uptake. As with some human leukemias and lymphomas, the disease produced by the Friend virus undergoes temporary remission on treatment with X-ray. In most cases the spleen in remission shows presence of many abnormal cells characteristic of the disease. Consistent with this latter observation, the P^{32} uptake of spleens in X-ray induced remission is higher than that of spleens from X-ray treated control animals(2). A more detailed examination of phosphorus metabolism of infected spleens was therefore undertaken. The effect of X-ray was also studied.

Methods. Virus and mice. Preparation of virus, method of infection, treatment with X-ray and mice used have been described(1). *Fractionation and analytical methods.* The general plan of experiments was as follows: About 20-40 infected and equal number of control mice were given 5-15 μc P^{32} i.p., and sacrificed 3 hours later, when spleens were removed, chilled and fractionated. Several experiments were done in which biochemical fractions of whole cells were separated by the Schneider, Schmidt-Thannhauser method(5). Results by this procedure were consistent with those obtained by subcellular fractionation combined with biochemical fractionation of subcellular elements, and will not be presented separately. Subcellular fractionation was performed as follows: spleens of mice killed by cervical fracture were perfused *in situ* with cold 0.85% NaCl, about 3-7 ml/mouse, by inserting 27-gauge short needle successively into several places in the spleen and gently expressing perfusing fluid from the syringe. The perfusate contained mostly red cells which otherwise would have formed the bulk of the nuclei preparation. Spleens were collected in ice cold 0.25 M sucrose and fractionated into subcellular particles using 0.25 M sucrose according to the method of Schneider and Hogeboom, as given in Umbreit, *et al.*(6), except that the nuclei were sedimented twice at 1000 rpm for 10 minutes in International rotor No. 253, and once at 2000 rpm for 10 minutes the mitochondria were sedimented in Servall SS-1 centrifuge at 9000 rpm for 10 minutes.*

Results. Experiments were performed on animals infected from 2 to 10 days. The earliest that metabolic changes were detected was 3 days after infection, when spleens had not yet enlarged. The data presented are restricted to 3-day, and 10-day infection, when marked spleen enlargement had occurred.

The effects of 3 days of infection on concentration of phosphorus in lipid and nucleic acid fractions of subcellular elements, and on uptake of P^{32} into these fractions are shown in Table I. With respect to phosphorus up-

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* In spite of perfusion of spleen, a great many red blood cells were in the nuclei fraction and some in the mitochondria fraction. It was sometimes necessary to resort to additional high and low speed cycles of centrifugation to reduce number of red blood cells. Judging by loss of DNA this additional washing resulted in loss of 20-40% of the original nuclei. Because of these losses the γ P/mg figures for nuclei and mitochondria represent only minimal values, but they serve to indicate relative values for infected and control tissues.

TABLE I. Alteration in Phosphorus Metabolism Associated with 3-Day Infection with Friend Virus.

	Infected		Control		Ratio:
	γP/mg	S.A., cpm/γP	γP/mg	S.A., cpm/γP	Spec. act. infected/Spec. act. control
Nuclei					
Lipid	.25	24	.24	25	.96
Nucleic acid	1.65	30	1.61	32	.94
Mitochondria					
Lipid	.18	31	.21	29	1.05
Nucleic acid	.02	142	.02	132	1.08
Microsomes					
Lipid	.45	22	.54	18	1.23
Nucleic acid	.36	22	.38	18	1.23
Supernatant					
Lipid	.08	78	.10	44	1.80
Nucleic acid	1.43	29	1.26	20	1.45

take the early effects (3 days) of the virus were restricted to stimulation of the microsome and supernatant fractions. In 2 other experiments the results were qualitatively the same although S.A.'s varied from experiment to experiment.

Table IIA presents comparable data for a 10-day infection. Specific activities of fractions from 10-day infected tissues are all higher than from uninfected ones, in all 4 experiments. In general, *concentrations* of several P fractions in infected spleens, particularly DNA phosphorus, were higher than those of control spleens, but not always so.

Data of Table IIA were obtained from half of group of mice. The other half was X-rayed on day 8 of infection, and given P^{32} and killed on day 10. Then average spleen weights were the same in X-ray infected and X-ray control animals, 0.065 g, as compared to 1.14 g for unirradiated infected and 0.184 g for unirradiated controls. Spleens of X-irradiated infected animals had about 1 log unit less virus than the non-irradiated ones. Data are summarized in Tables IIB and III.

It is clear that X-irradiation reduces concentration of nuclear nucleic acid in both infected and control tissues, and reduces P^{32} uptake into virtually all fractions in both groups. However, Table III shows that P^{32} uptake into fractions, particularly nucleic acid fractions, from infected tissues is more

TABLE II. Alteration in Phosphorus Metabolism Associated with 10-Day Infection with Friend Virus.

	A. Non X-rayed				B. X-rayed			
	Infected		Control		Infected		Control	
	γP/mg	S.A.	γP/mg	S.A.	γP/mg	S.A.	γP/mg	S.A.
Nuclei								
Lipid	.77	348	.71	127	.44	132	.64	109
DNA	2.3	400	2.7	273	.54	130	.37	168
RNA	1.8	395	1.2	300	.46	114	.43	116
Mitochondria								
Lipid	.46	363	.27	159	.64	219	.72	150
RNA	.22	309	.09	212	.10*	98*	.09*	194*
Microsomes								
Lipid	.56	389	.40	235	.38	237	1.01	154
RNA	.83	394	.31	132	.17	359	.23	322
Supernatant								
Lipid	.37	895	.27	258	.31	245	1.10	175
RNA	3.80	452	.71	277	.26	242	1.10	185

* Sample lost. These data from previous experiment.

TABLE III. Ratio of Specific Activities of Fractions from Infected Spleen to Those from Controls.

	Non X-ray	X-ray	% reduction by X-ray
Nuclei			
Lipid	2.74	1.21	56
RNA	1.32	.98	26
DNA	1.47	.77	48
Mitochondria			
Lipid	2.28	1.46	36
RNA	1.46 (1.82)*	(.51)*	(72)*
Microsomes			
Lipid	1.65	1.53	7
RNA	2.99	1.11	63
Supernatant			
Lipid	3.46	1.40	60
RNA	1.63	1.31	20

* Sample lost. These data from previous experiment.

strongly inhibited than those from controls.

Discussion. If one assumes that on day 3 of infection with Friend leukemia agent, most infected cells are in early phases of virus reproduction, then it appears that re: phosphorus metabolism, the first detectable effect† of the virus stimulates the microsome and subparticulate RNA and lipid. This could mean that virus RNA combines with microsomal particles to act as templates for manufacture of new virus materials, and is consistent with current thoughts on the role of microsomal particles and subparticulate elements in the synthesis of protein and RNA. (Previous work with fluorescent antibody(2) showed viral antigen in the cytoplasm of infected cells.) This hypothesis, however, is but one of several that could be used to explain the findings.

Animals infected for 10 days show increased P^{32} uptake into all biochemical fractions. It should be noted that the physical condition of spleens after 10 days infection was not the same for all experiments. Average wet spleen weights in 4 experiments were 0.766, 0.900, 1.14 and 1.46 g with ratios to control spleen weights ranging from 3½ to 7. There were varying degrees of engorgement with blood. In some experiments there was a 2-fold increase in DNA concentration as compared to controls, in some there was none. It may be that DNA concentration was high in spleens that were still growing rapidly, and decreased when growth reached a plateau, but there were not enough experiments to establish this.

X-irradiation reduced spleen size promptly and markedly in both infected and control mice. In experiment reported here in detail, infected spleens were reduced to the same size as controls, even though prior to irradiation, infected spleens averaged 6 times the size of controls. This reduction in infected spleen size after irradiation was a little more marked than usually seen.

It is worthy of mention that increase in P^{32} uptake/mg of tissue occasioned by infection was about as much after irradiation as before, even though irradiation caused a decrease in the ratio:

$$\text{Specific activity of biochemical fraction from } \frac{\text{Infected tissues}}{\text{Control tissues}} \text{ (Table III)}\cdot$$

This is understandable when one realizes that total radioactivity of a tissue depends upon both amount of phosphorus in different fractions, and their specific activities. Consider as an example the following hypothetical situation:

Biochemical fraction	Tissue 1 γP/mg	S.A.	(High spec. act. fractions) cpm/mg tissue
A	20	108	2160
B	10	279	2790
	Total cpm/mg tissue		4950
	Avg S.A.		165

Biochemical fraction	Tissue 2 γP/mg	S.A.	(Low spec. act. fractions) cpm/mg tissue
A	10	80	800
B	20	245	4900
	Total cpm/mg tissue		5700
	Avg S.A.		190

From the above, even with only 2 fractions to work with, distribution of total phosphorus between low and high specific activity fractions can lead the cpm/mg of tissue and average specific activity of Tissue 2 to be higher than those of Tissue 1 even though specific activities of individual biochemical fractions of

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† No effects were found prior to day 3, and in some experiments with weaker virus preparations, none were found even on day 3.

Tissue 1 are higher than those of Tissue 2. It is clear therefore that in some cases comparison of uptake of radioisotope by 2 whole tissues may give a misleading idea of their relative metabolic activities. As a corollary it follows that the more detailed the biochemical fractionation, the more meaningful are the data.

Summary. Some aspects of phosphorus metabolism of spleens of mice infected with Friend leukemia agent have been presented. A comparison has been made of concentrations of different phosphorus-containing cellular components as well as uptake of P^{32} into these components. Early in the infection, before significant spleen enlargement occurs, increase in uptake of P^{32} is restricted to microsomal and subparticulate lipid and nucleic acid. Later when spleen enlargement is marked, all phosphorus-containing fractions studied showed increased P^{32} uptake. X-irradiation, which caused marked reduction in spleen size, reduced P^{32} uptake into fractions of both control and infected spleens, with greater reduction in the infected ones.

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Stability Characteristics of Coe Virus.* (25575)

WILLIAM S. JORDAN, JR.
Depts. of Preventive Medicine and Medicine, School of Medicine, University of Virginia, Charlottesville

In preparation for studies of epidemiology of infections due to Coe virus, an agent isolated from the respiratory tract by Lennette and co-workers(1), it was important to investigate stability of this agent and to determine optimum conditions for handling and storing the virus. This paper presents evidence which indicates that Coe virus is relatively stable under varying conditions of pH and temperature.

Materials and methods. Tissue culture. Strain DMB cells derived from nasal mucosa were employed, using methods previously described(2). The nutrient fluid consisted of 40% pooled human serum and 60% Hank's balanced salt solution. Cells were washed 3 times with Hanks salt solution prior to use for virus growth. The maintenance fluid consisted of Scherer's MS 84.5%, chicken serum 8.0%, and tryptose phosphate broth 7.5% (3). Coe virus was supplied by Dr. E. H. Lennette after 6 passages in HeLa cells. Virus pools were prepared following 5 or 6 subsequent passages in DMB cells. *Infectivity Titrations.* Serial 1:3.2 ($10^{-0.5}$) dilutions were prepared in Hanks balanced salt solution, and 0.1 ml of each dilution was inoculated into each of 2 DMB cell culture tubes. Inoculated tubes were incubated in stationary racks at 37°C for 5 days. A culture was considered infected when 50% or more of cells had undergone specific cytopathic changes. Infectivity titer end-point expressed/0.1 ml inoculum was calculated as highest dilution of virus which infected one-half of tubes inoculated. *Alterations of pH.* Viral suspensions were adjusted to the desired pH with 0.5 N HCl or NaOH essentially as described by Ginsberg in a study of adenoviruses(4); in our study pH was measured with Beckman glass electrode meter. After adjustment to various hydrogen ion concentrations, the virus

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suspensions were allowed to stand at room temperature 30 minutes before readjustment to pH 7.3 - 7.5. The treated aliquots were then rapidly frozen and stored at –70°C until titrated simultaneously in DMB cells. Other methods of treatment and storage are described in relation to the results obtained.

Results. Localization of virus. To determine necessity for cell disruption when harvesting Coe virus infected cultures, the localization of virus at time of maximum cytopathic effect was investigated. Maximum CPE was obtained in 10 ml bottle in 48 hours. One-half of harvest was frozen and thawed 6 times, centrifuged at 2000 rpm for 10 minutes, and stored at –70°C. The supernatant fluid was removed from the other half after centrifugation of cells, and similarly stored. Cells were then washed 3 times in Hanks salt solution, and resuspended in an amount of maintenance fluid equal to volume of supernatant fluid removed. This suspension of cells was frozen and thawed 6 times, the debris centrifuged, and the fluid stored and titrated simultaneously with other aliquots. The titers were as follows: whole harvest after freezing and thawing, $10^{-6.0}$; supernatant fluid, $10^{-5.5}$; cells after freezing and thawing in new fluid, $10^{-5.5}$. Repetition of experiment gave the following results: titer of whole harvest, $10^{-6.0}$; supernatant fluid, $10^{-5.75}$; cells, $10^{-5.5}$; recombination of supernatant fluid and suspension of disrupted cells, $10^{-5.75}$. Since 25% to 50% of viral particles were still intracellular at time of maximum CPE, all subsequent harvests were subjected to freezing and thawing to disrupt the cells.

Stability after repeated freezing and thawing. After initial disruption cycle of 6 freezings and thawings, aliquots of Coe virus harvest were placed in sealed ampules and stored at –70°C. On successive days, the ampules were thawed at 37°C and refrozen in a dry ice-alcohol bath once each day, and returned to storage until completion of the tests. Repeated daily thawing of a virus suspension during 12 days of storage at –70°C resulted in no significant decrease in titer.

Stability on storage. Aliquots of virus suspensions were placed in sealed glass ampules, stored at 4°C or room temperature (22-26°C) for various periods of time, and then stored at –70°C until end of experimental period. Infectivity titrations were performed simultaneously on all 4°C samples; similarly, on all room temperature samples. Table I indicates that Coe virus retains its infectivity at these temperatures for 20 days.

TABLE I. Stability of Coe Virus at 4°C and Room Temperature (22°-26°C).

	Infectivity titer (log 10)			Infectivity titer (log 10)	
Days of storage	4°C	Room temp.	Days of storage	4°C	Room temp.
0	–6.0	–6.0	8	–5.5	nt
1	nt	–5.5	10	–5.25	–5.5
2	–5.5	nt	12	–5.25	nt
3	–5.5	–5.75	15	nt	–5.5
4	–5.5	–5.5	16	–5.25	nt
5	–5.5	–5.5	20	–5.5	–5.25

Stability at 37°C. Rate of inactivation of Coe virus when incubated at temperature used for propagation, *i.e.,* 37°C, was next determined. Undiluted culture fluid in sealed glass ampules was held at 37°C until assay. Although relatively stable when stored over a wide range in temperature, Coe virus deteriorated at 37°C. The geometric means of 2 experiments (Fig. 1) demonstrate that 90% of the virus was inactivated within 3 days, and that less than 1% of infectivity remained after 20 days.

Inactivation at 50°C. Rate of inactivation of Coe virus was determined by heating 1.0 ml volumes in a 50°C water bath for increasing periods of time. At each time interval a heated aliquot was immediately immersed in an alcohol-dry ice bath, and stored until infectivity titrations were done on all samples. Because of the persistence of infectious virus even after 30 minutes, the experiment was repeated 3 times, and the geometric means of infectivity end points calculated. Fig. II shows that more than 99.9% of virus was inactivated within 5 minutes. However, a small number of virus particles did survive for as long as 30 minutes. Virus infectivity could not be detected in the aliquot heated for 1 hour, nor was virus detected when this fluid was passaged undiluted and through one additional blind passage.

Inactivation by varying pH. Data relative

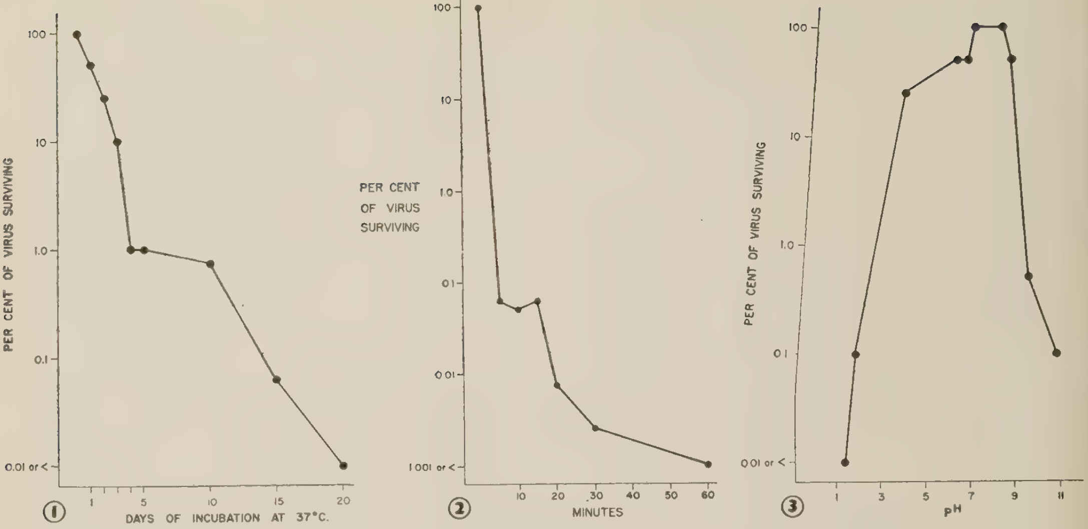

FIG. 1. Effect of continued incubation at 37°C on Coe virus in cell-culture maintenance mediums.

FIG. 2. Inactivation of Coe virus at 50°C.

FIG. 3. Inactivation of Coe virus by acid and alkaline pH changes.

to the stability of Coe virus when aliquots of a suspension were adjusted to varying pH values are plotted in Fig. 3. Coe virus was relatively stable at 22°-24°C between pH 6.0 and 9.0. Virus was inactivated outside of this range, but survival of virus even at pH's as low as 1.4 and as high as 10.8 emphasizes the stability of the infectious property.

Resistance to ether. Because of the importance accorded this characteristic in classifying viruses, the stability of Coe virus was tested by adding 0.4 ml of diethyl ether to 1.6 ml of virus suspension. The mixture was stirred at 4° for 15 hours, the ether removed following separation, and the virus titered. The titer of this suspension before treatment was $10^{-5.5}$; the titer after exposure to ether was $10^{-4.5}$. Thus, Coe virus was found to be ether resistant.

Discussion. Our data indicate that Coe virus is relatively resistant to inactivation by ether, by heat, and by pH changes. Pereira and Pereira(5) also found the agent to be ether resistant, and estimated size of Coe virus to be approximately 20 μ. Even though the virus does not grow in monkey kidney cells, these authors suggested that it be classed as an enterovirus. In terms of ether resistance and thermal stability, with deterioration of infectivity at 37°C, Coe virus does resemble a number of ECHO strains(6). It is equally true that the stability characteristics of Coe virus are similar to those of the adenoviruses (4), although Coe virus is more rapidly inactivated at 37°C.

Coe and adenoviruses grow in similar tissue culture cells—HeLa, KB, DMB, human amnion(1,5), but Coe virus dissociates much more readily from infected cells. When an adenovirus infected culture has undergone maximum cytologic alterations, only 2% to 6% of the virus detected in the cells is present in the fluid phase(7). Type 8 adenovirus is apparently an exception, for cell disruption is not necessary(8). Satisfactory titers of Coe virus can be obtained by harvesting the fluid phase alone, but the yield can be doubled by simultaneous disruption of the cells. The smaller Coe virus does not possess the common complement-fixing antigen shared by the adenoviruses; nor is Coe virus related to the myxoviruses or hemadsorption viruses(1). Since comparison of Coe virus with other animal viruses indicates that its stability to heat and pH alterations is of the same order as both the enteroviruses and adenoviruses(4,6), it is apparent that characteristics other than these must be used for classification.

The stability of Coe virus at 4°C, room temperature, and when maintained in the frozen state, should facilitate attempts to isolate this agent from human sources, and to define further its role as a respiratory pathogen.

Summary. Coe virus dissociates from infected cells, but 25% to 50% of virus is still intracellular at time of maximum cytopathic effect. The virus was relatively stable when exposed to repeated freezing and thawing, and to storage at –70°C, 4°C, and room temperature. At 37°C, 90% of the virus was inactivated within 3 days, and less than 1% of particles were infectious after 20 days. Coe virus is rapidly inactivated at 50°C, but a few particles survive 30 minutes at this temperature. Coe virus is ether resistant and resistant to inactivation at pH 6 to 9.

The excellent technical assistance of Eleanor Sturgill is gratefully acknowledged.

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Development of Myeloid (Chloro-) Leukemia in Thymectomized C3H Mice Following Inoculation of Lymphatic Leukemia Virus.* (25576)

Ludwik Gross
(With technical assistance of Yolande Dreyfuss and Lorraine A. Moore)
Cancer Research Unit, Vet. Admin. Hospital, Bronx, N. Y. City

Results of recent preliminary experiments suggested(1) that thymectomy renders mice of an otherwise susceptible strain (C3H) resistant to leukemogenic action of a highly potent lymphatic leukemia virus (passage A). This report deals with a) further observations of injected, and thymectomized mice for delayed development of leukemia; b) an attempt to restore susceptibility by subsequent implantation of normal mouse thymus, and c) the unexpected development of myeloid (chloro-) leukemia in some of the thymectomized mice inoculated with the lymphatic leukemia virus, and in one additional non-thymectomized mouse inoculated with the same virus and implanted with normal mouse thymus.

Methods. Passage A strain of leukemic virus initially derived from spontaneous Ak leukemia(2) and then passed serially by cell-free inoculations through newborn mice(3,4) was used. Now in its 21st serial passage, this virus induces consistently lymphatic leukemia in 85 to 100% of suckling C3H mice (Bittner subline) inoculated at less than 10 days of age. The average latency is about 2½ to 3½ months. Adult mice are also susceptible; up to 65% develop leukemia following intraperitoneal inoculation of the virus, but the latency is prolonged, varying usually from 4 to 6 months. Leukemic filtrates (Selas 02) of 20% concentration were prepared(3,4) from pooled leukemic organs from several C3H donors with primary, passage A virus-induced leukemia. All mice were of the C3H, or foster-nursed C3H(f) lines, both of Bittner substrain, bred in our laboratory. Spontaneous lymphatic leukemia in untreated mice of our C3H and C3H(f) colonies does not exceed 0.5%(4). We have never seen myeloid leukemia developing spontaneously in these mice.

Results. Resistance of thymectomized mice to inoculation of virus. It appeared previously that thymectomy either a) following, or b) preceding inoculation of passage A virus ren-

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TABLE I. Inoculation of Leukemic Filtrates into Suckling C3H Mice, Followed by Thymectomy.

Thymectomized[*]						Controls					
No. of mice inoc.[†]	No. dev. leuk.[‡]	Leuk. inc., %	Avg age leuk. dev., mo	No. dev. s.c. Sa	Avg age Sa dev., mo	No. of mice inoc.[†]	No. dev. leuk.[‡]	Leuk. inc., %	Avg age leuk. dev., mo	No. dev. par. tum.	Avg age tum. dev., mo
60	26[§]	43	10	5	9	85	79	93	3.6	2	4.8

[*] Avg age at thymectomy 32 days.

[†] 0.10 to 0.15 ml of 20% passage A fil. s.c. or i.p. Avg age at inoc., 3 days.

[‡] Avg age thymectomized mice died, or survived, with no signs of leukemia, 14.5 mo.

[§] 8 mice among these developed myelogenous leukemia at 12 to 14 mo of age.

dered mice resistant to development of leukemia. The reservation was made, however, that leukemia might still develop later in some thymectomized mice since the average age of surviving animals in group a) was only 4½ months(1). This indeed was the case. A considerable number of mice injected when newborn with virus and subsequently thymectomized, eventually developed leukemia. The incidence among controls was nevertheless substantially higher and leukemia developed earlier (Table I).

Of 60[†] mice inoculated with passage A leukemia virus at average age of 3 days, and thymectomized about a month later, *i.e.,* at an average age of 32 days, 26 (43%) developed leukemia at average age of 10 months. Of these, 8 developed myelogenous leukemia (chloroleukemia), in 4 the leukemias were unidentified, and in 14 lymphatic disease occurred. The remaining mice survived with no signs of leukemia to average age of 14.5 months. Of 85 litter-mate controls, inoculated simultaneously with the same filtrates, but not thymectomized, 79, or 93% developed lymphatic leukemia, after average latency of only 3.6 months. Thus, thymectomy performed after inoculation of the virus, inhibited development of the disease in some animals, or delayed it considerably in others. A different form of leukemia, hitherto not observed in our mice, that had been inoculated with the same virus, appeared in some of the thymectomized group. A few mice also developed subcutaneous fibro-myxo-sarcomas which were probably unrelated to inoculation of the leukemia virus. Passage A filtrates contain the parotid tumor virus(5), capable of inducing, among other tumors, also subcutaneous sarcomas. Thymectomy rendered most of these mice refractory to development of leukemia and prolonged their lives. Subcutaneous sarcomas developed, usually late, in some of these mice.

On the other hand when thymectomy was performed prior to inoculation of leukemia virus, no leukemia resulted even after prolonged observation. Thirty-nine[†] young adult C3H mice were thymectomized when 4 to 6 weeks old, and inoculated a few days later with passage A filtrate. None had developed leukemia, although all were observed until an average age of 15 months. Of 41 non-thymectomized control mice, inoculated simultaneously, when approximately 2 months old, with the same leukemic filtrate, 26 (63%) developed lymphatic leukemia at an average age of 8.7 months (Table II).

TABLE II. Effect of Thymectomy, Preceding Inoculation of Leukemic Filtrates, on the Incidence of Virus-Induced Leukemia.

Thymectomized				Controls				
Age at thym., days	No. of mice inoc.[*]	No. dev. leuk.	Avg surv. age, mo	No. of mice inoc.[*]	No. dev. leuk.	Leuk. inc., %	Avg age leuk. dev., mo	Avg surv. age, mo
55	39	0	15	41	26	63	8.7	14

[*] 0.5 ml of 20% passage A filtrate i.p. each. Avg age at inoc., 59 days.

[†] Several mice were added to this series since the last report was published(1).

TABLE III. Effect of Subcutaneous Implantation of Normal Thymuses on Susceptibility of Thymectomized C3H Mice to Leukemogenic Action of Passage A Filtrates.

	Filtrate only	Filtrate then thymectomy† followed by implantation of normal thymus‡	Filtrate then thymectomy‖
No. of mice inoc.*	36	20	5
No. dev. leuk.	35	18§	2
Leuk. inc., %	97	90	40
Avg age leuk. dev., mo	3.6	8.3	8.5

* Avg age at inoculation, 6 days.

† Interval between inoculation of filtrate and thymectomy, 1 mo.

‡ Interval between thymectomy and implantation of thymus, 7 to 30 days, avg 15 days.

§ 2 of these leukemias were myeloid (chloroleukemia).

‖ The No. of mice in this column is small, but is supported by similar data in Table I.

Attempt to restore susceptibility of thymectomized mice by implantation of normal thymus. Suckling C3H mice less than 8 days old were inoculated with passage A filtrate. After approximately 3 weeks these mice were divided into 2 groups. One group served as control. Mice of the other group were thymectomized. Seven to 30 days (average 15 days) after thymectomy, 20 thymectomized mice received implants of normal thymuses immediately after aseptic removal from young, approximately 3 weeks old, healthy, untreated C3H mice. Implants of whole thymuses from 2 donors were inserted through a small incision in skin of the back of each thymectomized mouse, and gently pushed into the subcutaneous tissue toward the chest. The wound was closed by one or 2 interrupted sutures. Of 36 control mice inoculated with the filtrate, 35 (97%) developed leukemia after average latency of 3.6 months. Of 20 mice inoculated with the filtrate, thymectomized later, and subsequently implanted with thymuses, 18 (90%) developed leukemia at an average age of 8 months (Table III). At least 2 of these leukemias were myeloid (chloroleukemia). In some of these mice a large tumor developed at site of subcutaneous implantation of normal thymuses; these mice also showed, however, evidence of generalized lymphatic leukemia, such as large spleens and livers, infiltrated with lymphoid cells, and large peripheral lymph nodes. In a small additional control group, 5 mice had been inoculated with the leukemic filtrate and were later thymectomized, but did not receive subsequent thymus implants. Only 2 of these developed leukemia after a latency of 8.5 months. These experiments suggest that susceptibility of thymectomized C3H mice to leukemogenic action of passage A virus could be restored by implanting into such mice normal thymuses from young, healthy C3H mice.

Development of myelogenous leukemia in mice that had been inoculated with passage A filtrates. Some C3H mice inoculated when newborn with passage A filtrates, and subsequently thymectomized, remained healthy for a time, but when approximately 12 months old, developed palpable spleens and enlarged peripheral lymph nodes. Autopsy of one of these leukemic mice revealed a green color of the peripheral lymph nodes and a more detailed examination, including microscopic studies of blood and other organs revealed myeloid leukemia. Within a few weeks 7 other mice with a similar form of leukemia were found among surviving filtrate-injected and subsequently thymectomized C3H mice. Myeloid leukemia also developed in 3 additional mice that had been inoculated with passage A virus; only 2 of these 3 mice had been thymectomized, but all 3 received subcutaneous normal C3H thymus implants.

C3H mice not inoculated with passage A filtrate, though thymectomized, did not develop myelogenous leukemia. In an unrelated experiment, 22 normal C3H mice not inoculated with leukemic filtrate were thymectomized when 3 to 4 weeks old. They were then observed without further treatment until they were 14 to 15 months old. None developed leukemia.

Morphology. All 11 mice with primary myeloid leukemia presented similar pathology. The moderately enlarged superficial and internal lymph nodes were in most instances of a characteristic light green color suggesting the diagnosis of chloroleukemia. The green color was particularly pronounced in 7 out of the

11 mice examined. Spleens were large, but livers were only slightly enlarged and of brown color. The number of peripheral white blood cells was increased (to 149,000/cu mm). Blood smears revealed in all 11 mice the presence of myeloblasts, myelocytes, metamyelocytes and unidentified large mononuclear cells; there were numerous myeloid cells and polymorphonuclear leukocytes in all phases of maturity, but relatively few lymphocytes. Nucleated red blood cells were also present; in one instance the number of nucleated red blood cells was considerable (103 nucleated RBC per 100 WBC). There was rapidly progressing anemia with a marked anisocytosis and polychromasia of erythrocytes. One leukemia could be identified as erythroblastic.

Transplantation of myeloid leukemia. Cell suspensions(2-4) from C3H donors with myeloid leukemia were inoculated intraperitoneally into young adult, 4 week old C3H mice. Leukemia developed in most of them after a latency of 2 to 6 weeks. The latency was shortened in subsequent transplantations. The characteristic green color of the superficial and internal lymph nodes was, however, considerably less pronounced in those mice that developed leukemia following transplantation of leukemic cells, than in initial donors with primary, virus-induced, leukemia. Some mice with transplanted leukemia revealed a varying amount of ascitic fluid and presence of multiple leukemic tumors in the peritoneal cavity resulting probably from the intraperitoneally implanted leukemic cells. Some mice had peripheral blood changes characteristic for myelogenous leukemia; in some of the C3H mice with transplanted myeloid leukemia, the number of white blood cells reached 500,000 and even 800,000/cu mm, with a large proportion of myeloblasts and immature myeloid cells. Frequently, however, lymphoid leukemia resulted, with a high proportion of lymphoblasts.

Discussion. The fact that removal of thymus conferred resistance to C3H mice to passage A leukemogenic virus was observed in our laboratory(1) and independently by Levinthal and her associates(6). Removal of the thymus prior to inoculation of the virus was most effective, since such thymectomized animals did not develop leukemia even after long interval of time. On the other hand, when thymectomy followed inoculation of the virus, some mice eventually developed leukemia, either lymphatic, or myelogenous. There was, however, a substantial difference between these 2 groups in age at which inoculation of virus was performed. Animals thymectomized first received the virus when approximately 4 or 5 weeks old. Mice of the other group received the virus shortly after birth, *i.e.,* at time of higher susceptibility. This could explain why a substantial number of mice in this group later developed leukemia. Levinthal and her associates did not observe development of leukemia in mice that had been inoculated with the virus and then thymectomized, but their experiments were terminated when the mice were only 7 to 9 months old(6). It is conceivable that at least some of their thymectomized mice could have developed leukemia later. It is rather difficult to remove completely both thymic lobes in an operative procedure requiring skill and speed to avoid hemorrhage and respiratory difficulties. We assume therefore, that in at least some of our mice that developed lymphatic leukemia in spite of thymectomy, parts of their thymic lobes might have been left after operation.

Implantation of a normal thymus into thymectomized animals restored their susceptibility to leukemogenic virus. In previous similar experiments by Law(7), Kaplan(8), Carnes(9), and their associates, implantation of normal thymus into thymectomized mice also restored their susceptibility to carcinogen or radiation-induced leukemia, respectively. More recently Miller(10) also observed that susceptibility of thymectomized C3H mice to inoculation of passage A leukemic virus could be restored even several months after thymectomy, by implantation of a normal, homologous thymus. These experiments suggest that thymus is not the only organ where the leukemic virus is stored. Animals that had been injected with the virus and were later thymectomized, apparently still carried the virus.

The most startling finding was the recent observation of development of myeloid leukemia in some of the thymectomized mice inoculated with passage A leukemia virus. That form of leukemia has not been hitherto observed in normal C3H mice receiving only passage A virus without additional treatment. In previous experiments several hundred newborn and suckling C3H mice had been inoculated with passage A filtrates. Up to 95% of the inoculated mice developed lymphatic leukemia. Over 70 blood counts were recently made on these mice. In some mice (not exceeding 20%) the disease was aleukemic. Peripheral blood examination revealed in the majority increased numbers of white blood cells (average 53,300/cu mm) predominantly of the lymphocytic series (average 65% of lymphocytes) with presence of lymphoblasts (average 7.1%) consistent with the diagnosis of lymphatic leukemia. Quite frequently, however, the blood picture became leukemic only in terminal phases of the disease.

Although there were not always significant peripheral blood changes, the plasma of these mice contained the virus. Filtrates of plasma from C3H mice with passage-A-virus-induced leukemia were almost as leukemogenic on inoculation tests as the highest concentrations of "organ filtrates"(3,4) of spleens, livers, thymic tumors, and mesenteric and peripheral lymph nodes from the same donors. This observation (unpublished) suggested that the leukemic virus is present in relatively high concentration in blood plasma of mice with passage-A-virus-induced leukemia.

Development of myelogenous leukemia in some C3H mice inoculated with passage A (lymphatic) leukemia virus is of considerable interest. Most of these mice were thymectomized after inoculation with the agent. One is tempted to speculate that removal of thymus had a significant relation to development of myelogenous leukemia in these mice. Another mouse also received passage A virus, but instead of having the thymus removed, received a subcutaneous implantation of an additional thymus from a homologous host. Here again there may be some obscure, perhaps indirect, relation between the implanted thymus and development of myelogenous leukemia.

It could be assumed that development of myelogenous leukemia was "spontaneous," and had no relation to injection of the virus. It would be difficult nevertheless to accept such an explanation. We have never seen spontaneous myelogenous leukemia among untreated mice of our C3H or C3H(f) colonies observed during the past 15 years. One could assume, on the other hand, that myelogenous leukemia developed in these mice as a result of inoculation of passage A leukemia virus. If so, a) the same virus might cause either lymphatic or myelogenous forms of leukemia, b) we may be dealing with a mixture of distinct leukemogenic viruses.

A similar phenomenon was observed in mice of the Ak strain, which carry, and transmit naturally from one generation to another, a leukemic agent(2) presumably identical with that contained in passage A filtrates(3). If left undisturbed, up to 90% of Ak mice develop leukemia spontaneously, as a rule lymphoid, when about 7 to 12 months old. Development of myelogenous leukemia in untreated Ak mice has been reported, but is exceedingly rare(11,12). We have not seen myelogenous leukemia among many hundreds of spontaneous Ak leukemias studied during the past 15 years.

Incidence of spontaneous leukemia in mice of the Ak strain could be considerably reduced by thymectomy(13). Furth observed, however, that among thymectomized Ak mice whose life was prolonged, several later developed myelogenous leukemia(12). Chloroleukemia was also observed in a mouse of the Ak strain(14), but that particular animal had been irradiated.

The first transmission of chloroleukemia in mice by cell transplantation was reported by Hall and Knocke(15). More recently, Graffi observed frequent development of chloroleukemia in mice inoculated with filtrates prepared from several transplantable mouse tumors. Only 10% of the myelogenous (chloro) leukemias observed by Graffi(16) could be transmitted by cell transplantation, but at least some of them could be transmitted by filtrates. The myelogenous leukemias we observed could be readily transplanted by cell graft, retaining their myelogenous blood morphology, or changing to lymphatic form. Attempts to transmit these leukemias to newborn and suckling C3H mice by filtrates are now in progress in our laboratory.‡

Burmester *et al.*(17) observed recently that when cell-free extracts prepared from a presumably pure strain of avian myeloblastosis A were inoculated into susceptible chicks, either myeloblastosis, visceral lymphoblastosis (lymphatic leukosis), renal adenocarcinomas, or osteopetrosis resulted. All combinations were observed except a concurrent development of myeloblastosis and lymphatic leukosis. In another series Burmester *et al.* observed that filtrates prepared from chicken erythroblastosis strain R induced either erythroblastosis, or visceral lymphomatosis. The fundamental question remains to be answered whether a single virus is capable of inducing different forms of chicken leukosis, or whether we are dealing with a mixture of distinct, although possibly related, leukemogenic agents. The problem is similar in the mouse.

Summary. 1. Thirty-nine C3H mice were thymectomized at 4 to 6 weeks of age and inoculated a few days later with passage A leukemic filtrates. None developed leukemia, although observed until they were 15 months old. Of 41 non-thymectomized adult control mice injected simultaneously with the same filtrates, 63% developed leukemia at 8.7 months average age. 2. Of 60 C3H mice inoculated with passage A leukemic virus at average age of 3 days and thymectomized a month later, 26 (43%) developed leukemia at 10 months average age. Among leukemic mice in this group, 8 developed myelogenous (chloro) leukemia. Of 85 litter-mate controls injected simultaneously with the same filtrates, but not thymectomized, 93% developed lymphatic leukemia at 3.6 months average age. 3. Myelogenous leukemia developing in some virus-injected and subsequently thymectomized mice could be readily transplanted to mice of the same strain, retaining its myelogenous form or changing to lymphoid. 4. Susceptibility of thymectomized mice to the leukemogenic action of passage A virus could be restored by subcutaneous implantation of thymuses from young normal C3H mice. Of 20 C3H mice injected with the leukemic virus, then thymectomized, and which subsequently received subcutaneous implants of normal C3H thymuses, 18 developed leukemia (2 of these were myelogenous), as compared with 35 of 36 non-thymectomized controls that had received the same filtrates.

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‡ As this manuscript goes to press, among the 117 C3H mice inoculated at an average age of 2 days with filtrates (Selas 02) prepared from myelogenous leukemias, 21 mice (18%) have thus far developed either thymic lymphosarcomas, or generalized lymphatic leukemia, at an average age of 3 months; a high proportion of lymphoblasts in peripheral blood was noticed in some of them.

Effect of ACTH on Permeability of Adrenal Cells to Sugar.*† (25577)

J. Eichhorn, I. D. K. Halkerston, M. Feinstein and O. Hechter
Worcester Foundation for Exp. Biology, Shrewsbury, Mass.

The view that the mechanism of adrenocorticotropic hormone (ACTH) action upon the adrenal cortex(1), like that of insulin in muscle(2), may involve regulation of cellular permeability processes is being systematically investigated in this laboratory. We briefly reported(3) that D-xylose is excluded from a major fraction of cell water of the adrenal gland in hypophysectomized rats, and that ACTH administration increases D-xylose distribution in adrenal tissue, but not in muscle. Insulin, on the other hand, increases D-xylose distribution in muscle but has no effect in the adrenal. In this paper, detailed results on the effect of ACTH upon adrenal cell permeability to D-xylose, mannitol, sucrose and inulin are presented.

Methods. The studies were conducted using male rats of Sprague-Dawley strain, weighing 120-170 g, with the pituitary either intact or removed 42-48 hr earlier. Animals were anesthetized with avertin (1 ml of 2% solution/100 g body wt, intraperitoneally), and the renal pedicle was ligated close to the hilum using care not to occlude adrenal blood flow. Immediately thereafter, one of the following C^{14}-labeled substances was injected subcutaneously: D-xylose-1-C^{14} (Nat'l. Bureau of Standards), mannitol-1,6-C^{14} (Volk Radiochemicals), sucrose-U-C^{14} and inulin-carboxyl-C^{14} (both from New England Nuclear). Unless otherwise stated, hormonal preparations were injected immediately prior to administration of tracer substance. Corticotropin A (Armour), corticotropin α_b (Li), and β-corticotropin (Bell) were given intravenously (I.V.) in 0.3 ml N/100 HCl-saline; insulin (Iletin, Lilly) intraperitoneally in glucose-saline (using 100 mg glucose/100 g to prevent hypoglycemia), controls for this group received 25 mg glucose/100 g; corticosterone was given in divided dose in 10% ethanol, 50% I.V. and 50% subcutaneously; and bovine growth hormone (Li), I.V. in saline. At varying intervals after administration of radiotracers, the rats were anesthetized with nembutal, blood drawn into a heparinized syringe from the abdominal aorta, and plasma separated immediately. Simultaneously, the animals were exsanguinated and tissues removed, blotted, and weighed. Adrenals were weighed after removal of capsules. In various experiments, samples of diaphragm, gastrocnemius, liver, epididymal fat, and testes were excised. Tracer substances were extracted from tissues by boiling in water for 15 minutes(4,5) and extracts and diluted plasma (1:50) were deionized with the mixed exchange resin Amberlite MB3 (Lamotte Model X) which had been dried at 80° for 20 hr (in control experiments no loss of tracers occurred, including carboxyl labeled inulin). Extracts were planchetted and counted in a flow gas chamber.

Results are presented in terms of volume of distribution of test substance in total tissue, expressed as percent of total tissue wet weight in equilibrium with plasma concentration. Table I presents volumes of distribution of D-xylose in adrenals at 60, 90 and 120 min. after administration to functionally nephrectomized, hypophysectomized or intact rats. Fig. 1 plots individual distribution values shown in Table I against plasma concentration of D-xylose. Over a wide range of plasma levels of D-xylose, distribution values of both intact and hypophysectomized groups remain quite constant within each group. Moreover, an essentially steady equilibrium exists throughout 60′ to 120′ independent of whether the pituitary is intact or removed. These data are consistent with the idea that transfer of a "non-utilizable" sugar into adrenal tissue is by diffusion into a compartment where entry is directly proportional to external concentration. Average volume of distribution of D-xylose in adrenals of intact rats, which

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TABLE I. Volumes of Distribution of D-xylose* in Adrenal Tissue at 60, 90, and 120 Minutes after Tracer Administration in Intact or Hypophysectomized Rats.

Time interval (min.)	Intact pituitary (%)	Hypophysectomized (%)
60	59.0 ± 2.0 (7)	28.4 ± 1.0 (15)
90	61.2 ± 2.2 (22)	33.0 ± 1.1 (19)
120	62.9 ± 2.0 (7)	31.0 ± 3.1 (6)

* D-xylose-1-C^{14}, 800 to 3500 μg/100 g body wt.

had been stressed by the operative procedure, was 59-63% over 60-120 min. covered in Table I. After hypophysectomy, distribution values fell markedly to 28-33%, indicating that xylose is excluded from a large fraction of adrenal tissue in absence of the pituitary.

Table II presents volumes of distribution at 90 min. of various radiotracers in adrenals of functionally nephrectomized hypophysectomized rats treated with various corticotropins, as well as other hormones. An increase in adrenal distribution of D-xylose was achieved in hypophysectomized rats with exogenous ACTH: corticotropin A increased distribution from 30% to 51% ($p = <.001$), corticotropin α_b (Li) to 42% ($p = <.001$) and 36 mU/100 g of Bell's β-corticotropin to 36% ($p = <.05$). Table II also shows that insulin and corticosterone in doses tested had no effect on xylose distribution, despite a concurrent increase with insulin in xylose distribution in diaphragm from 33% ± 3 (S.E.) to 115% ± 46, and in gastrocnemius from 18% ± 1 to 50% ± 5. Li growth hormone given alone (10 μg) or with submaximal dosages of ACTH (30 μg growth hormone, 36 mU β-corticotropin) did not affect adrenal distribution of xylose. Table II also shows that spaces occupied by sucrose, mannitol, and inulin were not identical, as would be expected if all were true measures of extracellular volume. The inulin space was not affected by ACTH administration nor by factors operating in stressed rats with intact pituitaries. Using

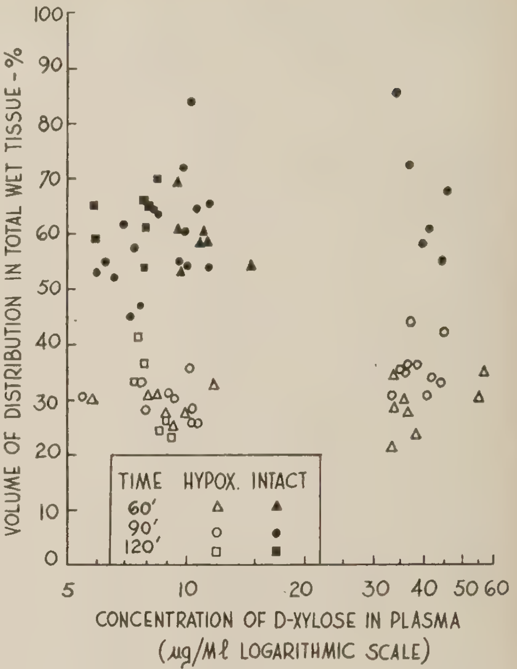

FIG. 1. Volume of distribution at various plasma concentrations of D-xylose in adrenals of functionally nephrectomized hypophysectomized or intact rats 60, 90, and 120 min. after administration.

TABLE II. Effect of Various Hormones on Volumes of Distribution of Tracer Substances in Adrenals of Functionally Nephrectomized Rats.

	Treatment (with dosage/100 g body wt)	Vol of distribution* of various radiotracers† in adrenal D-xylose	Inulin	Sucrose	Mannitol
	Pituitary intact	59.3 ± 2.5 (16)	14.0 ±.4 (9)	18.2 ±2.4 (6)	27.2 ± 2.3 (5)
Hypophysectomized	HCl-saline	30.0 ± 1.0 (9)	14.9 ±.6 (14)	15.3 ±.7 (6)	22.4 ± 1.8 (5)
	Corticotropin A (375 mU)	51.1 ± 3.8 (8)	14.0 ±.1 (6)	20.1 ±.7 (8)	30.6 ± 3.2 (5)
	α_b corticotropin, Li (540 mU)	41.7 ± 2.3 (14)			
	β ACTH, Bell (36 mU)	35.6 ± 2.2 (11)			
	Glucose (25 mg)	26.6 ± 1.2 (5)			
	Insulin (1 U) + glucose (100 mg)	29.2 ± 2.0 (6)	14.6 ±.3 (4)		
	Alcohol	29.5 ± 1.6 (6)			
	Corticosterone (700 μg)	29.0 ± 1.5 (6)			

* Determinations were made 90 min. after administration of tracers and hormones. Values are the mean, stand. error, and No. of observations.

† D-xylose-1-C^{14} (specific activity, 2.26 μc/mg), 800 μg given per 100 g body wt; inulin-carboxyl-C^{14} (S.A. 1.37), 1240 μg; sucrose-U-C^{14} (S.A. 7.41), 202 μg; mannitol-1,6-C^{14} (S.A. 8.47), 165 μg.

inulin as a measure of extracellular volume, corticotropin A increased the percentage of intracellular water occupied by xylose from 28% to 66%.[‡] No significant changes in volumes of distribution of xylose or inulin in diaphragm, gastrocnemius, testes, or epididymal fat were noted after hypophysectomy or after ACTH.

Discussion. These studies established that D-xylose is excluded from a large fraction of adrenal cell water in hypophysectomized rats, and that ACTH facilitates xylose entry, specifically into the adrenal cell. Absolute values for xylose distribution in adrenal cortex alone cannot be stated at this time, but considering absence of medullary responses to ACTH by other criteria, it might be expected that ACTH would not affect the medulla in present studies. Demonstration of an ACTH effect on xylose uptake does not require 90 min. interval routinely employed, since ACTH significantly increased distribution of xylose in the adrenal within 20-30 min.[§] The time course of response was not pursued further, since at sufficiently brief intervals extremely small increases in availability of a utilizable sugar, like glucose, could produce metabolic results not measurable in terms of xylose distribution.

This response of the adrenal to ACTH is similar to that of skeletal and diaphragmatic muscle to insulin. In absence of insulin, a very limited entry of xylose into muscle cells occurs, suggesting(5,6,7) that a barrier exists which excludes xylose from a major fraction of total intracellular water. If one assumes that all cells within tissues are homogenous in their permeability characteristics, it appears that both insulin and ACTH alter intracellular "barriers" and thus permit entry of D-xylose into much larger compartments in respective target organs. On the other hand, if tissues contain cells with membranes of differing permeabilities, it is possible that some of the cells may be freely permeable to xylose in absence of hormone and ACTH or insulin affect only remainder of cells.

If D-xylose reflects permeability behavior of glucose in the adrenal, as appears to be the case in muscle(2,5,6,7), the significance of these findings to adrenocortical physiology may be widespread. An increase in availability of glucose to adrenocortical cells might be conceived as responsible for many phases of ACTH action, including steroidogenesis, growth, and ascorbic acid release. Without speculative elaboration, the findings demonstrate a specific *in vivo* response of the adrenal to ACTH which must be considered in any explanation of the mechanism of ACTH action. Finally, the similarity of effects on permeability of insulin and ACTH in their respective target organs suggests that these hormones may in this respect have a common mode of action.

Summary. Volumes of distribution of D-xylose and of substances which measure extracellular fluid volume were determined in adrenal and other tissues of nephrectomized rats. Hypophysectomy markedly reduced distribution of D-xylose in intracellular water of the adrenal, and exogenous ACTH increased xylose entry specifically in the adrenal to levels approaching those found in stressed animals with intact pituitaries. Attention is drawn to possible relation of these findings to the mechanism of ACTH action.

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[‡] Intracellular water was assumed to be the difference between total tissue water (69.8% ± .9 (S.E.), 12 observations) and inulin space.

[§] Renal ties and tracer administrations were carried out 60-70 min. before ACTH, so that total interval for tracer equilibration was 90 min.

Effect of Hydroxyzine* on Urine Flow in the Dog.† (25578)

William P. Blackmore
Dept. of Pharmacology, University of Texas Southwestern Medical School, Dallas

Antidiuresis implicating liberation of the antidiuretic hormone has been well established following administration of several agents which depress the central nervous system(1). More recently, reserpine(2) and chlorpromazine(3), both tranquillizing agents which also exert an effect on the nervous system, have been reported to inhibit water diuresis. While conducting preliminary experiments on another tranquillizing agent, hydroxyzine, a decrease in urine flow was noted in dogs. As a result this communication presents findings concerning the effect of hydroxyzine on urine flow and its mechanism of action. Sanchez and Davis(4) recently reported antidiuresis in hydrated rats which had received hydroxyzine by intraperitoneal injection.

Methods. All experiments were performed on trained unanesthetized dogs. Normal and diabetes insipidus dogs were used. Neurohypophysectomies were performed by the temporal approach in which a high stalk section was made.‡ The diabetes insipidus animals were not used until a month following operation, and daily urine output was in excess of 2000 ml. Two types of experimental procedures were employed. In the first series, experiments were conducted similar in principle to that described by de Bodo(5). Animals were kept in metabolism cages and hydrated with 50 ml/kg of water. At the end of 3 hours the bladder was emptied by catheterization and the same amount of water was again given. Excretion of the second water load was used for evaluating the effect of hydroxyzine. The drug was injected 40 minutes after the second water load was administered and urine excreted during the 40 minutes interval subtracted from the total water load since the drug had no effect on this fraction. Hydroxyzine was administered intravenously in a dose of 5 mg/kg over a 5 to 10 minute interval. In the second series of experiments the animals were hydrated with 30 ml/kg of water 1 hour prior to experiment. Throughout the experiment glucose 5% in water was infused at a constant rate of 3 ml/minute. Following a control period, hydroxyzine was infused intravenously in a dose of 5 mg/kg over a 30 minute period. Urine was collected for an additional 2½ hours following cessation of hydroxyzine infusion. Determinations of glomerular filtration rate, renal plasma flow, osmolar and "free" water clearance were obtained by methods previously described(6). Statistical analysis of the data was performed by the method of Mather(7) in which values obtained during the hydroxyzine infusion and each subsequent hour were compared to control values.

Results. Table I shows the effect of hydroxyzine on urine flow during water diuresis in normal dogs. Values given are average per cent of volumes obtained in each experiment; figures in parenthesis represent minimum and maximum amount of the water load excreted.

TABLE I. Effect of Hydroxyzine (5 mg/kg) on Water Diuresis in Normal Dogs.

Dog No.	No. of exp.	Type of exp.	% of second water load excreted at 3 hr
1	3	Control	98 (93, 102)
	3	Drug	67 (61, 74)
2	4	Control	96 (94, 100)
	4	Drug	75 (65, 83)
3	2	Drug	74 (76, 73)
4	3	Control	99 (96, 103)
	3	Drug	71 (59, 80)
5	2	Drug	69 (72, 66)
6	2	"	66 (61, 71)
7	1	"	67
8	1	"	74
9	3	Control	93 (94, 105)
	3	Drug	77 (74, 80)
10	2	Drug	86 (82, 90)
11	3	Control	95 (91, 98)
	3	Drug	73 (62, 80)

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‡ We are grateful to C. J. Shepler, D.V.M., for performing the operations.

TABLE II. Effect of Hydroxyzine (5 mg/kg) on Glomerular Filtration Rate, Urine Flow, Osmolar and Free Water Balance.

GFR, ml/min.				V, ml/min.				C_{osm}, ml/min.				C_{H_2O}, ml/min.			
C	I	PI 1 hr	PI 2 hr	C	I	PI 1 hr	PI 2 hr	C	I	PI 1 hr	PI 2 hr	C	I	PI 1 hr	PI 2 hr
60	59	56	57	4.5	4.0	1.6	1.5	.7	.7	1.1	.8	3.8	3.3	.5	.7
71	71	59	65	5.6	6.0	2.8	3.0	1.4	1.2	1.1	1.2	4.2	4.8	1.7	1.8
69	79	79	73	4.9	6.0	2.8	2.8	.7	.7	.9	1.0	4.2	5.3	1.9	1.7
91	98	95	84	6.3	5.4	4.7	1.9	1.1	1.0	1.2	1.4	5.2	4.4	3.5	.5
67	71	72	74	4.2	4.2	3.5	3.5	.7	.6	.6	.6	3.5	3.6	2.9	2.9
84	83	84	78	5.4	5.5	4.4	3.1	.9	.7	.8	.7	4.5	4.8	3.6	2.4
59	59	68	64	5.0	6.7	4.1	1.7	.4	.5	.9	.6	4.5	6.2	3.2	1.1
55	58	53	56	5.0	4.8	2.5	2.3	.9	.9	.7	.8	4.1	3.9	1.8	1.5
64	59	63	66	4.7	4.6	2.9	2.8	1.1	1.2	1.0	1.0	3.6	3.4	1.9	1.7
68.9*	70.8	69.9	68.5	5.1	5.2	3.2	2.5	.9	.8	.9	.9	4.2	4.4	2.3	1.6
±3.9†	±4.6	±4.6	±3.1	±.2	±.3	±.3	±.2	±.09	±.08	±.06	±.09	±.2	±.3	±.3	±.2

* Mean. † S.E.

Volume of urine excreted in 3 hours by the control animals was an average of 96.2%. In the experiments where hydroxyzine was administered, a definite decrease in urine output occurred, the average being 72.6% of the second water load during the 3 hour period. These results indicate that hydroxyzine decreased urine output in dogs undergoing water diuresis, thus raising the possibility that this effect may have occurred either because of hemodynamic changes or a specific effect of hydroxyzine on water reabsorption. Results of the experiments where hydroxyzine was infused for 30 minutes are illustrated in Table II. The drug also produced a decrease in urine flow. This occurred only during the period following cessation of hydroxyzine. No change in glomerular filtration rate occurred in these experiments $P > 0.05$). Similarly, hydroxyzine caused no change in renal plasma flow. These findings indicate that the decrease in urine flow was not the result of any hemodynamic changes, suggesting a specific antidiuretic effect. Determination of osmolar clearance revealed no significant change throughout the experiment ($P > 0.05$); however, a significant fall ($P < 0.05$) in "free" water clearance occurred during the period following cessation of hydroxyzine. These results indicate that hydroxyzine influenced only "free" water reabsorption. It is of interest to note that the antidiuretic effect did not occur immediately as is generally observed with other antidiuretic agents. This delayed onset could imply that antidiuresis did not occur until sufficient time had elapsed for hydroxyzine to cross the blood brain barrier. However, the fact that some degree of tranquillization occurs clinically in most individuals within 15 to 30 minutes, when given orally, indicates the drug does cross the blood brain barrier rapidly, and would not explain the delay in onset. Another possibility is that the response is not due to hydroxyzine *per se*, but rather to a metabolic product of hydroxyzine. Unfortunately, no chemical test is available for determination of hydroxyzine in tissues so this delayed onset can not be explained.

The effect of hydroxyzine on urine flow in diabetes insipidus dogs is summarized in Table III. Following infusion of hydroxyzine there was no change in urine flow in contrast to the decrease noted in normal animals. These re-

TABLE III. Effect of Hydroxyzine (5 mg/kg) on Urine Flow in Diabetes Insipidus Dogs.

Dog	Exp. No.	Control	Hydroxyzine	Post-infusion 1 hr	Post-infusion 2 hr
		ml/min.			
1	1	4.4	3.9	4.0	5.4
	2	5.0	4.6	6.1	5.3
	3	4.3	3.8	4.7	4.9
	4	3.7	3.3	4.8	4.7
2	1	4.7	5.6	4.6	5.2
	2	3.0	4.8	4.3	6.1
	3	4.3	4.8	5.4	4.8
	4	5.0	4.1	4.7	5.4
3	1	3.8	4.2	4.8	4.1
	2	5.4	4.9	5.2	5.9
	3	4.3	4.6	4.0	4.5
	4	3.6	3.9	4.0	3.4
Mean		4.3	4.4	4.8	5.0
S.E.		±.2	±.2	±.2	±.3

sults indicate that hydroxyzine stimulated release of the antidiuretic hormone in normal dogs, otherwise similar results would have been obtained in both types of animals. In addition, measurement of osmolar and "free" water clearance in 4 experiments in diabetes insipidus dogs showed no change from the control values.

Summary. Intravenous administration of hydroxyzine to unanesthetized dogs in doses of 5 mg/kg produced a decrease in urine flow following cessation of the drug in absence of any hemodynamic changes. In contrast to response in normal dogs there was no change in urine flow in diabetes insipidus animals. These results indicate hydroxyzine stimulated release of the antidiuretic hormone under conditions described here.

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Response to Stress: Differing Responses of Atherosclerotic and Normal Dog to Electric Shock or Cold.*† (25579)

SHAWN SCHAPIRO‡ AND JESSIE MARMORSTON
Inst. for Medical Research, Cedars of Lebanon Hospital, Los Angeles and School of Medicine, University of Southern Calif.

Inability to adapt successfully to stressful situations may be a factor in development of cardiovascular pathology. Although the role of the autonomic nervous system and hypothalamic-pituitary-adrenal axis in response to stress has received intensive study, the interrelations between this system and cardiovascular pathology are virtually unexplored. This report describes differing response of atherosclerotic and normal (non-atherosclerotic) dogs to stresses of electric shock and cold.

Methods. Thirty-five dogs were used. Twenty-one (9 beagles and 12 mongrels) were made atherosclerotic by maintenance on standard thiouracil and cholesterol diet (consisting of 50 mg thiouracil/kg body weight and kibble containing cholesterol prepared with ether solvent to provide daily cholesterol intake of 750 mg/kilo body weight) as previously reported by this group(1). These dogs were maintained on the atherogenic regimen for 6 months to 2 years. The remaining 14 dogs (9 beagles and 5 mongrels) served as controls and received standard kibble diet. *Electric shock.* The 21 atherosclerotic dogs and 14 normal dogs were subjected to electric shock. Each dog was anesthetized with sodium pentobarbital, after which 5 shocks were administered across frontal lobes within 15 minutes by electric shock machine delivering 450 milliamperes of 0.7 sec. duration. Urine samples were collected hourly by catheter for 3 hours prior to and 3 hours following shock treatment. Urine was analyzed for corticoids by the Heard-Sobel method(2) and for creatinine by the method of Bonsnes and Tausky(3). *Cold stress.* Four normal dogs and 4 atherosclerotic dogs were placed in metabolic cages. A minimum of three 24-hour urine collections at room temperature served as controls for each dog. Cold stress consisted of

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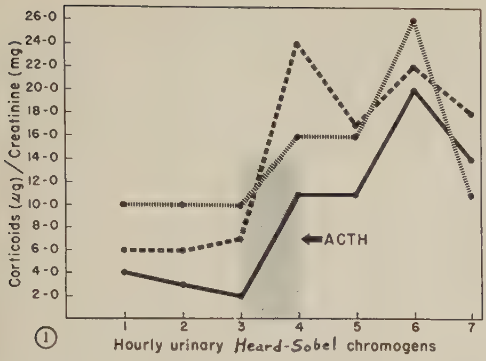
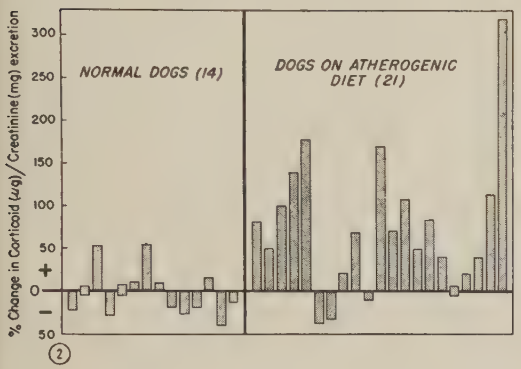
† We would like to acknowledge the help of Dr. Harry Sobel during the very early phases of this work and we thank LeRoy Krause for assistance throughout the study.

‡ Present address: Neuropsychiatric Hospital, Veterans Admin. Center, Los Angeles, Calif.





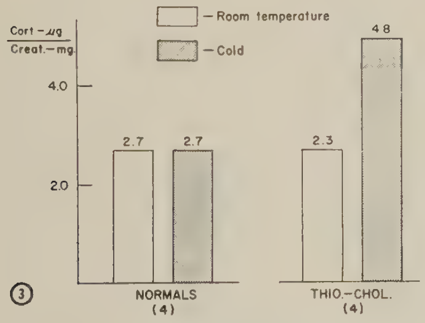


FIG. 1. Effect of ACTH infusion on corticoid excretion by 3 normal mongrels.

FIG. 2. Effect of electric shock stress on corticoid excretion of normal dogs and dogs on atherogenic diet.

FIG. 3. Urinary corticoids and creatinine of normal and thiouracil-cholesterol treated dogs before and after cold stress.

maintenance of dogs in "cold room" at 0° for 24 hours. *ACTH infusion.* To determine whether output of urinary corticoids as identified by Heard-Sobel method furnishes a measure of ACTH-dependent activity, 3 normal dogs were infused with ACTH (one unit/kg in 100 cc saline solution) for an hour, and urine specimens were collected before, during, and after infusion period. In each case, infusion of ACTH caused an increase in corticoid/creatinine ratio (Fig. 1) indicating that methods used to determine urinary corticoids were detecting ACTH-dependent activity.

Results. With Electric Shock. Dogs on atherogenic diet showed a greater response than normal dogs to stress of electric shock, as indicated by higher excretion of corticoids relative to creatinine (Fig. 2).

With Cold Stress. In no instance was urinary corticoid activity of the normal dog increased by exposure to cold, Contrariwise, the 4 atherosclerotic animals each exhibited a marked increase in adrenocortical activity (Fig. 3).

Discussion. Although basal output of normal and atherosclerotic dogs was approximately the same, our study demonstrates that dogs maintained on an atherogenic diet for 6 months to 2 years exhibit a greater response to stress produced by electric shock or by cold than occurs with normal dogs. Our experiments do not indicate the locus of altered hypothalamic-pituitary-adrenal response to stress. Some controvery exists as to whether the adrenal gland of the hypothyroid animal is more responsive to ACTH and to stress than the adrenal gland of normal animal. Hess and Finerty(4), using ascorbic acid depletion in the rat, interpret their observations to indicate that this is the case. However, according to Eik-Nes and Brizzee(5) the adrenal of the thyroidectomized dog has a lower basal corticoid output and is less responsive to ACTH. It is also possible that thiouracil exerts an effect upon the hypothalamic component of stress response. In support of this, Martini demonstrated that propylthiouracil produces distinct histologic change in cells of the anterior hypothalamus and neurohypophysis(6).

A hypothyroid condition may be present in human atherosclerosis. For review see (7). In line with this observation, serum cholesterol levels are usually elevated in myxedema(8). Similarly, in the dog the hypothyroid state is necessary to produce atherosclerosis(9). The serum cholesterol level apparently bears some relationship to nervous apprehension or anxiety(10-13). Myasnikov(14) found evidence in rabbits that drugs acting directly upon the central nervous system exert a modifying influence on serum cholesterol and on development of experimental atherosclerosis. Raab(15) recently reviewed literature showing relationship of neurohumoral agents to atherogenesis, while Vedeneeva(16) obtained histologic and electrocardiographic evidence of development of myocardial infarctions several days after administration of a large dose of adrenalin. These observations tending to implicate the neuroendocrine system in pathological deposition of lipid material in vascular walls may be related to older findings of Hausberger(17) more recently confirmed by Sidman and Fawcett(18) that lipid mobilization from, and deposition in, fat depots depends upon intact innervation to these areas.

The influence of the nervous system upon development of experimental atherosclerosis has not been studied as extensively as the possible importance of endocrine and dietary factors. The effect of chronic cardiovascular pathology upon integrative functions of the neuroendocrine system is virtually unexplored.

Our results indicate that, at least in the dog, such pathology—produced by prolonged period on an atherogenic diet—is associated with an altered hypothalamic-pituitary-adrenal response to stress. Further work is needed to clarify the site and mechanism of this altered reactivity.

Summary. Dogs maintained on an atherogenic diet to 2 years exhibited a greater response to electric shock or cold stress than did normal control animals.

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Antimicrobial Effect of Abalone Juice. (25580)

C. P. LI
Division of Biologics Standards, N.I.H., Bethesda, Md.

Meat of the abalone (*Haliotis refescens*) (1) has been a common food item in China for many generations. A familiarity with the characteristics of this sea animal, which has access to a wide variety of organic material of biological origin led the author to consider it as a potentially valuable source of material in which to search for an antimicrobial agent. The investigation reported here indicates that there is, indeed, an effect of abalone upon growth of *Staphylococcus aureus* and to some extent also upon the course of experimental poliomyelitis in mice although the mechanisms involved are obscure.

TABLE I. Effect of Abalone on Experimental Poliomyelitis in Mice in 7 Trials.

Virus type	MID_{50}/mouse	Days treatment started before infection	Treated		Control	
			Paralysis or death[*]	%	Paralysis or death[*]	%
III	32	3	12/20	60	18/19	95
"	32	3	14/19	74	19/20	95
"	3	3	7/20	35	12/20	60
"	3	7	17/50	34	32/50	64
"	3	7	11/50	22	24/50	48
II	5	2	7/12	58	12/12	100
I	1	7	18/50	36	29/50	58

[*] No. paralyzed or dead/No. inoculated.

Materials and methods: *Assay of abalone with experimental poliomyelitis.* Abalone purchased as the canned product[*] was used. Each can contained about 200 ml of juice and about 250 g of meat. The juice used for experiments was removed aseptically, pooled and stored at 4°C. After a few days storage the juice separated into 2 layers; the upper two-thirds was brownish yellow in color and fairly clear, and the lower one-third was grayish white in color and quite cloudy. Sometimes the upper layer became a gelatinous semi-solid. The whole juice was warmed and the 2 layers well mixed before use. Mouse and tissue culture adapted type I (Mahoney) type II (Lansing) and type III (Leon) polioviruses all maintained in our laboratory and grown in monkey kidney tissue culture were used. General purpose Swiss mice (obtained from Animal Production Section, DBS, NIH), weighing 12-15 g were inoculated with one to 32 mouse infective doses in 0.02 ml of type I or III virus by intraspinal (IS) route and observed for 2 weeks. For type II virus, mice weighing 17-22 g were inoculated with 5 mouse infective doses in 0.03 ml by intracerebral (IC) route and observed for 3 weeks. Treated mice were fed with abalone juice. Approximately 1 ml of the juice was given to each mouse/day placed on whole wheat bread or mixed with commercial mouse diet. The control mice were fed with regular mouse diet. *Assay of Abalone with Staphylococcus aureus.* At beginning of present study, the canned abalone juice was tested for inhibiting effect on *Staphylococcus aureus* by absorbent paper disc method and results were inconsistent. Toward end of the work, frozen fresh abalone[†] was secured and some brownish juice was obtained upon thawing. This brownish quice was centrifuged and heated to 70°C for 30 minutes before use. A penicillin sensitive strain of *Staphylococcus aureus*, FDA 209, was used as test organism. The test was carried out as follows.[§] Ten-fold dilutions of a 24 hour culture of *Staphylococcus aureus* in buffered saline were made and a loopful of each dilution was inoculated into a Wassermann tube containing 1.8 ml of nutrient broth with 0.2 ml of the heated abalone juice. The same dilutions were also inoculated into control tubes without abalone. After incubating at 35°C for about 18 hours, 0.1 ml from each tube was spread over surface of nutrient agar plate. The plates were then incubated overnight and colonies on each plate counted.

Results. The results on experimental poliomyelitis in mice are summarized in Table I. In treated mice the rates of paralysis and death were lower, average incubation period was longer and paralysis was less severe than in control mice. All experiments showed a similar curve of accumulative percentage of paralysis or death. Examples are shown in Figs. 1 and 2.

The effect of abalone on growth of *Staphylococcus aureus* is illustrated by 2 sample experiments shown in Table II. Both experiments were carried out with the penicillin sensitive strain, FDA 209. They were repeated

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[*] Calmex Brand of Marine Products Co., San Diego, Calif. or Brand of Chong Kee Co., N. Y.

[†] Frozen fresh abalone was kindly supplied by Dr. H. G. Orcutt, Bureau of Marine Fisheries, Calif.

[‡] This culture was kindly supplied by Dr. A. L. Schade of National Institute of Allergy and Infectious Diseases.

[§] This method of testing was suggested by Dr. C. W. Hiatt of this laboratory.

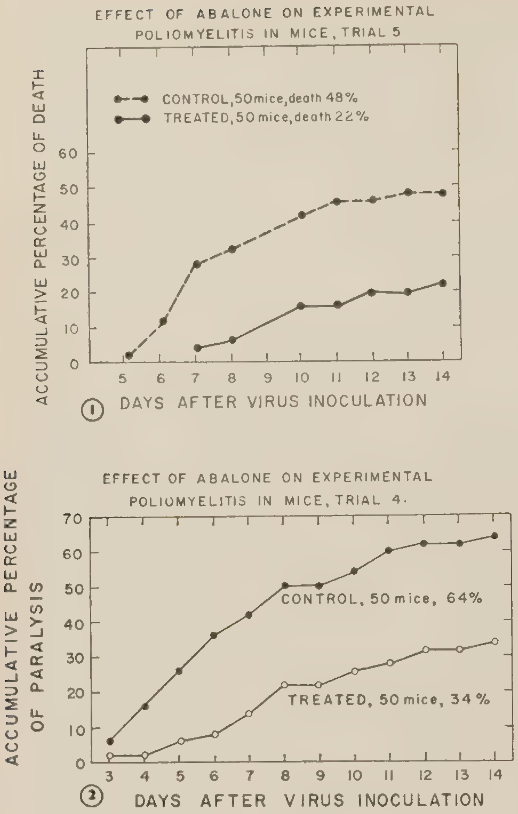

FIG. 1 and 2.

TABLE II. Effect of Abalone Juice on Growth of *Staphylococcus aureus*, FDA 209 Strain.

		Colonial growth from 0.1 ml of 18 hr broth cultures inoculated with one loopful of serial dilutions					
Trial	Abalone in broth	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Control*
1	+	36†	8	0	0	0	0
	—	Con.‡	Con.	Con.	Con.	Con.	0
2	+	84	4	0	0	0	0
	—	Con.	Con.	Con.	Con.	TN§	0

* Control: not inoculated.

† Besides 36 colonies there were 2 small patches of growth.

‡ Confluent growth.

§ Colonies too numerous to be counted but not confluent.

several times and consistent and similar results were obtained. In some experiments the crude juice from frozen fresh abalone was heated to 95°C for 45 minutes or dialyzed against water overnight and its effect on *Staphylococcus aureus* was not impaired.

Discussion. In reviewing the literature for antiviral(2-4) and bacterial agents, it was noticed that efforts have been largely concentrated in the field of microbial products. It is now felt necessary to shift toward some other material. Because of abundance and variety of organic materials in sea water, and the known ability of gastropods to filter and concentrate suspended solids of their aquatic milieu, certain sea animals such as abalone, may be regarded as a potential source in the search for antimicrobial agents. Our experiments demonstrated clearly antimicrobial action of crude fresh abalone juice against *Staphylococcus aureus*, although the effect of canned abalone juice against experimental poliomyelitis in mice was only moderate. Thermostability and nondialyzability of the agent suggested that it is not an ordinary antibiotic. One of the chief advantages of its possible use for therapeutic purpose is that it is edible as a food, and therefore non-toxic. Further work on purification and concentration is in progress.

Summary. Commercial canned abalone juice given to Swiss mice orally possessed sparing effect against intraspinal infection of type I and III poliovirus and intracerebral infection of type II poliovirus. Crude fresh abalone juice also possessed marked inhibitory effect against *Staphylococcus aureus*.

The author expresses thanks to Dr. C. W. Hiatt of this laboratory for enthusiastic suggestions, criticisms and other help, and also to Messrs. W. G. Jahnes, E. C. Martino, J. L. Rogers and E. W. Harvey for technical assistance.

‖ Dialysis was kindly carried out by Dr. A. K. Saz of Nat. Inst. of Allergy and Infectious Diseases.

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Stop Flow Analysis of Aldosterone and Steroidal Antagonist SC-8109 on Renal Tubular Sodium Transport Kinetics.* (25581)

ARTHUR J. VANDER,[†] WALTER S. WILDE AND RICHARD L. MALVIN[‡]
Dept. of Physiology, School of Medicine, University of Michigan, Ann Arbor

Recently(1), using the technic of stop flow analysis(2,3), we demonstrated a distal site of action of aldosterone. After adrenalectomy, the distal tubule was not able to reduce sodium concentration to minimum value achieved during stop flow in normal dogs. We have since noted that, on occasion, normally low distal sodium concentrations can be attained in the adrenalectomized animal only when associated with extremely low plasma sodium concentrations. The present paper presents experiments designed to study this relationship between plasma and distal tubular sodium concentrations in normal, in adrenalectomized and in dogs treated with synthetic steroid, SC-8109, 3-(3-oxo-17β-hydroxy-19-nor-4-androsten-17α-yl) propionic acid γ-lactone (Searle), which appears to block the renal effects of aldosterone and desoxycorticosterone(4-6).

Methods. Adrenalectomy and corticoid therapy of dogs prior to stop flow procedure. All experiments were performed on male or female mongrel dogs weighing 9-20 kg. These dogs can be divided into 4 groups, according to method of preparation utilized prior to performance of stop flow procedure: *Group 1*: Bilateral adrenalectomy was performed aseptically under pentobarbital anesthesia, either in a single operation by means of midline incision, or in 2 stages, performed one week apart and utilizing flank incisions. Cortisone acetate, 100 mg intramuscularly, was given at time of operation and on the day preceding. Thereafter, dosage of cortisone was gradually lessened, final maintenance dosage varying from 10-25 mg/day. These dogs were given normal diet supplemented with 100 mM Na/day. No animals were employed until at least one week after bilateral adrenalectomy. They were given usual dosage of cortisone 2 hours before starting the stop flow procedure. *Group 2*: These dogs were prepared exactly as in Group 1, except that all cortisone was discontinued 2 days prior to stop flow analysis. *Group 3*: Normal dogs were given a normal diet with no steroid. *Group 4*: Normal dogs were given SC-8109, approximately 0.75 mg/kg intramuscularly, 2 hours before beginning stop flow and every 2 hours during stop flow procedure. *Stop flow procedure.* All animals were anesthetized with 30 mg/kg of Na pentobarbital injected intraperitoneally. Through a small flank incision, one ureter was catheterized with polyethylene tubing pushed well up into the renal pelvis and tied firmly into place. A constant intravenous infusion of creatinine, PAH, and 18% mannitol in Ringer's Solution was supplied at 10 ml/min. Duration of occlusion varied from 4-6 min. Blood samples were taken from catheter in the femoral artery at onset and on release of occlusion. No ureteral occlusions were performed until urine flow had stabilized at about 10 ml/min. Three different experimental patterns were then followed, each on separate occasion: *Pattern 1*: Occlusions were performed at 15 min. intervals, varying length of occlusion from 4-6 min. *Pattern 2*: After completion of first occlusion, plasma sodium concentration was raised rapidly by intravenous administration of 2-3 g NaCl in 50 ml water for 5 minutes. After waiting 10 additional minutes, the next occlusion was performed, and the process repeated with another salt injection. *Pattern 3*: After completion of several occlusions, 7-40 μg of aldosterone were given intravenously for 15 min. Further occlusions were then performed after waiting 2-3 hrs. Aldosterone was given only to adrenalectomized dogs. *Analytical methods* were as follows: sodium was analyzed with Beckman direct-reading flame photometer,

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* Supported by Am. Heart Assn.; Life Insurance Medical Research Fund; Nat. Inst. of Arthritis and Metabolic Diseases, U.S.P.H.S. The compound, SC-8109 kindly provided by G. D. Searle and Co., Chicago, and aldosterone by U.S.P.H.S.

[†] Fellow, Am. Medical Educ. Fn.; Borden award, 1959.

[‡] Lederle Fellow.

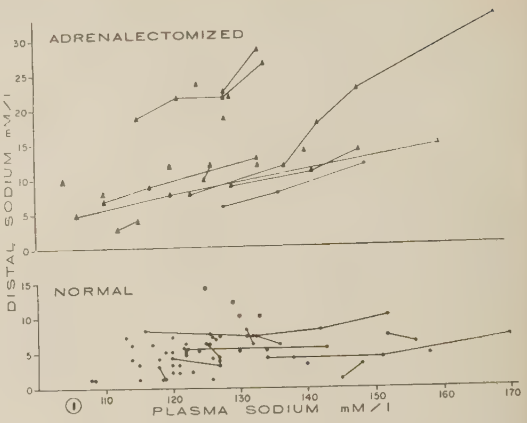
PAH according to Smith, Finkelstein, Aliminosa, Crawford, and Graber(7), creatinine by method of Bonsnes and Taussky(8).

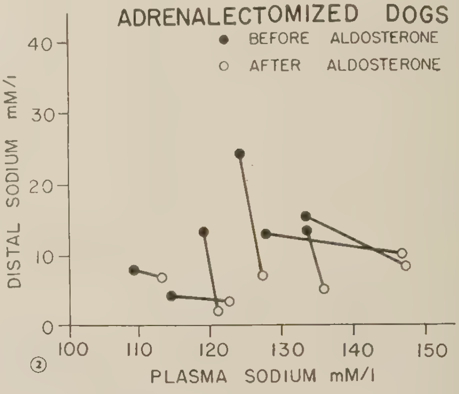
Results. Proximal tubule. It is possible, using calculations described previously(9), to determine volume of fluid reabsorbed from the proximal tubule during period of ureteral occlusion. The computed additional proximal reabsorption of sodium and water during ureteral occlusion was the same in adrenalectomized dogs both before and after administration of aldosterone.

Distal tubule. The minimum point on the downward inflection to the left of sodium curve is thought to represent best the lowest Na concentration developed in a maximum number of low sodium distal segments during stop flow(3). This minimum distal Na concentration is plotted vertically in Fig. 1 against increasing plasma Na along horizontal axis. In the normal animal, the distal tubule is capable of lowering sodium concentration almost to zero during stop flow, as demonstrated previously(1). The minimum sodium concentration achieved appears independent of plasma sodium concentration (except perhaps at extremely high values).

The effects of adrenalectomy upon this pattern (Fig. 1) are striking. Even at very low plasma sodium concentrations, the distal concentrations achieved during ureteral occlusion are higher than normal. Moreover, there is a direct relationship between concentrations of sodium in plasma and in the distal tubule during occlusion. This indicates that adrenalectomy has reduced maximal sodium concentration gradient which can be maintained across distal tubular cells. As plasma sodium rises, the minimal sodium concentration attained during ureteral occlusion also rises. Even if duration of occlusion is prolonged from 4 to 6 minutes, the distal sodium concentration attained during stop flow is not changed, indicating that maximal distal tubular sodium reabsorption occurs within the first 4 minutes of ureteral occlusion.

There was no difference between the group of animals treated with cortisone pre-operatively and the group in which all steroid therapy had been withdrawn 2 days pre-operatively.





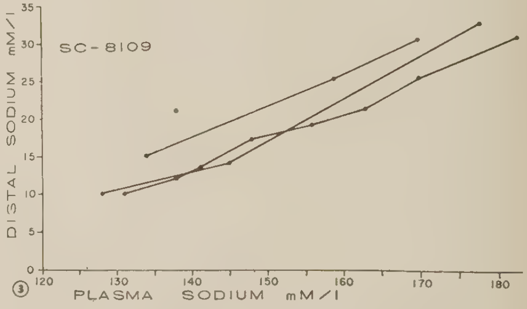


FIG. 1. Relationship between plasma Na concentration and the minimal distal urinary Na concentration developed during ureteral occlusion in normal and adrenalectomized dogs. Lines connect points derived from a single dog as plasma Na was elevated.

FIG. 2. Effect of aldosterone upon maximal distal tubular Na reabsorption during ureteral occlusion on adrenalectomized dogs. Lines connect points determined for a single dog as plasma Na was elevated.

FIG. 3. Relationship between plasma Na concentration and the minimal distal urinary Na concentration developed during ureteral occlusion in dogs treated with SC-8109. Lines connect points determined for a single dog as plasma Na was elevated.

The effects of aldosterone on adrenalectomized dogs are demonstrated in Fig. 2. Administration of aldosterone restored ability of the distal tubule to lower sodium concentration even in presence of elevated plasma sodium concentration.

Fig. 3 demonstrates the effects of SC-8109 on normal dogs. It is evident that inhibition of distal tubular sodium reabsorption produced by these large doses resembles that produced by bilateral adrenalectomy.

These studies further establish the distal tubule as site of action of aldosterone in sodium reabsorption. They also emphasize the importance of maintaining high plasma sodium whenever assays are made of possible blocking agents.

Summary. In normal dogs the distal tubule can lower Na concentration almost to zero during stop flow, the minimal concentration achieved being independent of plasma Na concentration. In adrenalectomized animals, minimal distal concentrations achieved are not as low. As plasma Na is increased, minimal distal Na concentration also increases in direct proportion, indicating that adrenalectomy reduces maximal Na concentration gradient which can be maintained across distal tubular cells. Results in adrenalectomized and in SC-8109 treated dogs are identical. Administration of cortisone to adrenalectomized dogs does not repair this defect, but aldosterone restores ability of the distal tubule to lower Na concentration, even in presence of elevated plasma Na. Aldosterone when given to adrenalectomized dogs did not alter proximal Na reabsorption, as this can be estimated by stop flow data.

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Activator of Plasminogen and Protease from Various Cell Cultures and Present in Poliomyelitis Vaccine. (25582)

SAMUEL BARON AND EUGENE V. BARNETT (Introduced by George A. Hottle)
Dept. of HEW, P.H.S., Nat. Inst. Health, Div. of Biol. Stds., Bethesda, Md.

Previous reports described and characterized an activator of plasminogen produced by KB cells and both an activator of plasminogen and a protease produced by Rhesus monkey kidney (MK) cells(1-4). The present study was undertaken to determine which cell cultures produce these 2 substances and whether they were present in poliomyelitis vaccine prepared from supernatant medium from MK cell culture.

Materials and methods. Cell cultures used are described in Table I. Supernatant medium from cell cultures was obtained as previously described(3). Each supernatant medium was assayed 2 to 5 times for activator and protease using skim milk as substrate(3). Commercially prepared poliomyelitis vaccine, with preservative, without preservative and after dialysis was assayed for activator and protease.

Results. Production of activator and protease by various cell cultures. Cell cultures fell into 3 groups, depending upon production of activator, activator and protease, and detection of no activity in the supernatant medium. As seen in Table I of 21 continuous

TABLE I. Production of Activator and Protease by Various Cell Cultures.*

Produces activator			Produces activator, protease			No activity		
Cell line	Reference	Growth medium	Cell line	Reference	Growth medium	Cell line	Reference	Growth medium
KB†	Eagle	199-10% CS	MK†	(primary)	199-2% CS	LAC†	Baron, Rabson	199-10% HS
KB‡	"	BME 10% HS	RK†	"	"	MS†	Tytell, Melnick	199-10% CS
HeLa†	Gey	199-10% CS	HK†	"	199-10% CS	HH‡	Girardi	BME 10% HS
HeLa S-3‡	", Puck	BME 10% HS				Detroit-98‡	Berman, Stulberg	"
HeLa‡	Gey	"				Detroit 6‡	*Idem*	"
HC‡	Chang	BME 20% HS				D-189‡	Leighton	"
Monkey heart†	Salk	199-10% CS				Human embryonic intestines‡	Henle	"
RK†	Drew	199-20% CS						
MAF‡	Gray	BME 10% HS				Liver‡	Chang	"
Monkey thyroid primary†	Hare	BME 20% HS				Sarcoma 180‡	Foley	BME 10% ES
						J-111‡	Osgood	BME 10% HS
						Bovine kidney‡	Mai	BME 10% CS
						Bovine skin & muscle‡	"	"

* Abbreviations are as follows: CS = calf serum, HS = human serum, HC = human conjunctiva, BME = basal medium, Eagle, MK = monkey kidney, RK = rabbit kidney, HK = human kidney, ES = horse serum, HH = human heart, Mai = Microbiological Associates, Inc.

† Grown at DBS, NIH, by Dr. J. G. Crawford and George Gardner.

‡ Obtained from Microbiological Associates, Bethesda, Md.

cell lines examined, 9 produced only activator (2 to 256 units/0.2 ml), none produced protease, and 12 had no activity as activator or protease. Different lots of 3 strains of HeLa cells produced varying amounts of activator and some lots produced none. Of the 4 primary cell lines tested, 3 of kidney origin produced both activator (32 to 4096 units/0.2 ml) and protease (2 to 256 units/0.2 ml). Production of protease by kidney cell culture was reported earlier(2,4).

Activator of plasminogen and protease in poliomyelitis vaccine. Eighty-three lots of poliomyelitis vaccine were tested for activator and protease. Activator alone was present in 29 lots, activator and protease was present in 8 lots while 46 lots had no activity. The amounts of activator and protease in vaccine were generally lower than the amounts in MK supernatant medium. Results were similar whether the lots of vaccine tested contained preservative, were without preservative or were dialyzed.

To determine if presence of these proteolytic materials in vaccine affected antigenic potency, monkey potency ratios(5) of all lots tested were reviewed. There was no relationship between presence of proteolytic materials and antigenicity for monkeys.

Discussion. The data indicate that activator for plasminogen and protease were produced by many continuous and primary cell cultures. Simultaneous production of activator and protease was limited to 3 primary cell cultures which originated from kidneys of 3 different species. Production of activator and protease and possibly other biologically active materials by cell cultures may be of value for identification and classification of these cultures.

The presence of active biological substances in poliomyelitis vaccine indicates that these substances resist destruction by inactivation process to which the vaccine is subjected. Similar substances, as tryptic leucoprotease, have been demonstrated to be highly resistant to formalin(6). Heat stability studies show that temperatures to which vaccine is subjected for inactivation would not destroy these proteolytic substances. These data show that active materials other than virus antigen occur in poliomyelitis vaccine. The high levels of protease inhibitors in sera and body tissues (7) make the presence of proteolytic substances in vaccine of doubtful clinical significance. Similarity of activator from tissue slices and activator produced by cell cultures has been noted(3). It has been proposed that fibrinolytic conditions *in vivo* are due to the activator from tissue slices(8). Most of the activator produced by cell cultures is released into the supernatant medium. It is possible that cells *in vivo* may release activator into extracellular fluid and be responsible for fibrinolytic conditions *in vivo*.

Summary. Assays of 25 metabolizing cell culture lines disclosed that 13 of these released an activator of plasminogen resembling tissue activator. Of 4 primary cell lines tested the 3 of kidney origin produced both activator and protease. Assay of a specific cell culture fluid, commercially prepared poliomyelitis vaccine, disclosed the presence of both activator and protease in many lots of vaccine.

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Enzymatic Determination of Glucose-6-phosphate in Tissue Extracts Containing Glutathione and Other Interfering Substances.* (25583)

H. T. NARAHARA† AND PINAR OZAND‡ (Introduced by F. E. Hunter)
Dept. of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo.

Enzymatic reduction of triphosphopyridine nucleotide (TPN) with glucose-6-phosphate dehydrogenase (G-6-PDH) can be used for quantitative determination of glucose-6-phosphate (G-6-P)(1). However, most preparations of G-6-PDH are contaminated with other enzymes which interfere with accurate determination of G-6-P, especially when carried out in crude tissue extracts. In the present report are described conditions under which G-6-P may be measured in tissue extracts by use of sufficiently purified G-6-PDH preparations and the selective inhibition of both hexokinase and glutathione reductase.

Methods. Three preparations of yeast G-6-PDH were gifts from C. F. Boehringer and Sons (Mannheim). A pure crystalline barium salt of G-6-P from Sigma Chemical Co. was converted to potassium salt before use. Oxidized glutathione (GSSG) was prepared from reagent grade glutathione (GSH) of Fisher Scientific Co. by the method of Rall and Lehninger(2). Formation of reduced TPN (TPNH) was followed at 340 mμ in Beckman DU Spectrophotometer, using 1 ml cell with light path of 1 cm. The reaction was carried out at room temperature in *tris*-(hydroxymethyl)aminomethane (Tris) buffer at pH 8.3 with sufficient G-6-PDH to oxidize 0.03 μmole of G-6-P in about 1 to 3 minutes in the presence of inhibitors to be described below. The blank solution contained Tris and the inhibitors but no enzyme. The reaction was initiated by addition of G-6-P or tissue extract to experimental and blank solutions, which brought the volumes to 1 ml. The initial baseline reading, corrected for dilution by substrate, was subtracted from the stable final reading, and the change in optical density was divided by 6.22 to obtain μmoles of TPNH

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† Special Research Fellow of U.S.P.H.S.

‡ Fellow of Rockefeller Fn.

TABLE I. Assay of G-6-P in Presence of GSSG.

		Increase in optical density					TPNH (μmole)
GSSG	$HgCl_2$-GSH	2 min.	4 min.	6 min.	8 min.	10 min.	2 min.
—	—	.200	.200	.200	.200	.200	.032
+	—	.138	.104	.084	.072	.065	.022
+	+	.201	.200	.200	.200	.200	.032

Reaction mixture: 0.1 M Tris pH 8.3, G-6-PDH, TPN 0.15 μmole, G-6-P 0.032 μmole. GSSG 0.024 μmole and $HgCl_2$-GSH reagent 0.1 ml added as indicated. Means of duplicate assays.

produced(3). The completed blank mixtures had an optical density of about 0.040 when read against distilled water. No significant activity of 6-phosphogluconic dehydrogenase, phosphoglucoseisomerase or phosphoglucomutase was associated with the G-6-PDH preparations; glucose-1, 6-diphosphate and $MgCl_2$ were added when testing for phosphoglucomutase. A change in optical density of 0.001 or less in 10 minutes in presence of appropriate substrate was considered insignificant. $HgCl_2$-GSH reagent was prepared by mixing a freshly made solution of 3.2 x 10^{-3} M GSH with equal volume of 3 x 10^{-3} M $HgCl_2$; 0.1 ml of this mixture was used/reaction cell. Tissue extracts for G-6-P analysis were prepared by grinding frozen liver from non-fasted rat, or saline suspension of rat erythrocytes, or frozen frog leg muscles in cold 1 N $HClO_4$ with sand in a mortar. Precipitated proteins were centrifuged, and excess $HClO_4$ removed from supernatant fluid by adjusting the pH to 7.2 with KOH, chilling on ice, and centrifuging off the $KClO_4$. About 0.005 ml of a 0.02% aqueous solution of phenol red was added as indicator/ml of $HClO_4$ extract; in control studies, addition of phenol red to a neutralized extract did not alter results of the G-6-P determination.

Results. When determination of G-6-P was carried out in presence of added GSSG, there was a rapid initial reduction of TPN accompanied by slower reoxidation of TPNH formed (Table I), which indicated contamination with glutathione reductase. A mixture of GSH and $HgCl_2$ could inhibit glutathione reductase rather selectively. Concentrations of $HgCl_2$ and GSH as low as 10^{-6} M inhibited completely the glutathione reductase activity associated with G-6-PDH, but these compounds at concentration of 1.5 x 10^{-4} M did not appreciably inhibit G-6-PDH, and produced only about 40% inhibition of hexokinase. $HgCl_2$ alone did not produce the same type of differential inhibition, and an excess of ethylenediaminetetraacetic acid (EDTA) did not reverse or prevent inhibition of glutathione reductase. The $HgCl_2$-GSH reagent, when mixed with Tris buffer at pH 8.3, retained its ability to inhibit glutathione reductase after standing 5 hours at room temperature; longer intervals were not tested.

When extracts of rat liver and erythrocytes were analyzed for G-6-P, there was interference by the relatively high concentration of GSSG in these tissues (Table II). The $HgCl_2$-GSH reagent effectively prevented reoxidation of TPNH, as indicated by the stable and higher optical density values obtained. An extract of frog leg muscle showed no tendency to reoxidize TPNH, and no significant difference in G-6-P content was found when

TABLE II. Determination of G-6-P in Tissue Extracts.

		Increase in optical density					
Tissue	$HgCl_2$-GSH	1 min.	2 min.	3 min.	4 min.	5 min.	10 min.
Liver	—	.030	.014	.006	.003	.0	.0
”	+	.045	.045	.046	.045	.045	.045
Erythrocytes	—	.005	.001	.0	.0	.0	
”	+	.011	.012	.012	.011	.011	
Muscle	—					.095 ±.0009	.095 ±.0008
”	+					.096 ±.0006	.096 ±.0006

Reaction mixture: Tris, TPN, and G-6-PDH as in Table I, plus 0.1 ml 0.1 M EDTA adjusted to pH 8.3 with KOH. Extracts correspond to 14.2 mg liver, 29.3 mg muscle or 0.013 ml packed RBC. Means of duplicate assays for liver and RBC; quadruplicate determinations for muscle, ± stand. error of mean.

muscle extracts were assayed with or without $HgCl_2$-GSH reagent (Table II).

Two of the G-6-PDH preparations had significant hexokinase activity, which poses a problem, since glucose and ATP are present in most tissue extracts. However, when $MgCl_2$ was omitted and EDTA added, hexokinase was completely inhibited without appreciable loss of G-6-PDH activity(4).

To test recovery of G-6-P, a measured amount was added to a $HClO_4$ extract of muscle prior to neutralization; a 70% increase in G-6-P content was observed in quadruplicate determinations, and this agreed within 1% with the theoretical value.

Discussion. Steiner and Williams(5) recently showed that GSSG in liver extracts interferes with enzymatic assay of G-6-P. They were able to remove the GSSG with Dowex 50. G-6-P was separated from free glucose in their extracts by precipitation with barium and alcohol. The present procedure makes these additional steps in determination of G-6-P unnecessary.

The important but separate problem of obtaining tissue extracts without stimulating glycogenolysis has not been considered in this presentation.

Summary. A method is described for measurement of glucose-6-phosphate in tissue extracts by use of glucose-6-phosphate dehydrogenase. Interference resulting from contamination of this enzyme with glutathione reductase was abolished by addition of a mixture of $HgCl_2$ and glutathione. Hexokinase, another contaminating enzyme which interfered with the determination, was inhibited by removing Mg ion. Phosphoglucomutase and phosphoglucoseisomerase were not present in significant amounts.

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Regulation of Cholesterol Mobilization in Mice by Bile Acids.* (25584)

WILLIAM T. BEHER, WILLIAM L. ANTHONY AND GIZELLA D. BAKER
Edsel B. Ford Inst. for Medical Research, Henry Ford Hospital, Detroit, Mich.

In a study of effects of several bile acids on cholesterol levels and synthesis rates in mice (1), 2 of these substances—cholic and hyodeoxycholic acids—had definite but opposing effects. Cholic acid caused substantial increase in liver cholesterol levels, which triggered 50% decrease in rate of acetate-1-C^{14} incorporation into liver total cholesterol. On the other hand, hyodeoxycholic acid decreased liver cholesterol, and greatly increased synthesis rate. Since previous experiments(2,3) had shown that cholic acid greatly retards mobilization of accumulated mouse liver cholesterol, it seemed of interest to test the effect of hyodeoxycholic acid on the same parameter to see if it would effectively increase the rate of cholesterol mobilization.

Procedures. Two hundred twenty Webster strain female, albino mice, weighing 18-20 g, were placed on a synthetic basal cholesterol free diet (CFD)(4) for 2 weeks, and then divided into 2 groups. Fifty mice received unsupplemented basal diet (CFD), while remaining 170 mice were placed on diet of CFD plus 0.5% cholic acid and 1% cholesterol. At end of 3-week cholesterol build-up, 10 normal mice and 10 mice from the build-up group were sacrificed for liver and carcass choles-

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TABLE I. Mobilization of Accumulated Liver Cholesterol in Mice Fed Cholic and Hyodeoxycholic Acids, as a Function of Time.

	Liver total cholesterol, mg/g liver				
Group	0 time	1 wk	2 wk	3 wk	4 wk
A. Normal—CFD*	6.59 ± 1.56	6.74 ± 1.61	6.84 ± 1.11	7.02 ± 1.56	7.93 ± 1.00
B. Regression—CFD	18.7 ± 4.06	20.3 ± 5.40	15.3 ± 2.90	10.5 ± 2.45	7.67 ± 2.17
C. *Idem* + 0.5% cholic acid	18.7 ± 4.06	19.4 ± 3.03	19.0 ± 3.54	18.4 ± 3.70	18.1 ± 2.60
D. " + 0.5% hyodeoxycholic acid	18.7 ± 4.06	8.46 ± 6.17	3.28 ± 1.05	3.21 ± .51	3.57 ± .93
E. " + 0.5% cholic acid + 0.5% hyodeoxycholic acid	18.7 ± 4.06	9.56 ± 5.23	6.21 ± 2.12	6.36 ± 2.30	3.98 ± .55

Statistical comparison of values:

Groups	1 wk	2 wk	3 wk	4 wk
A and B	$P < .01$	$P < .01$	$P < .01$	$P = .76$
A and C	"	"	"	$P < .01$
A and D	$P = .52$	"	"	"
C and E	$P < .01$	"	"	"

* CFD = cholesterol-free diet.

± numbers are stand. dev.

terol determination. The remaining 40 normal (Group A) mice were continued on CFD, while mice with elevated cholesterol were divided into 4 regression groups of 40 mice each: Group B, regression controls, were returned to CFD; Group C received CFD plus 0.5% cholic acid; Group D, CFD plus 0.5% hyodeoxycholic acid; and Group E, CFD plus 0.5% cholic acid and 0.5% hyodeoxycholic acid. At weekly intervals one-fourth of mice from each group were sacrificed, their livers removed and analyzed for total cholesterol(3). Stomachs and intestines were removed and cleared of all ingested material, then hydrolyzed together with the carcass and blood by refluxing for 3 hours in 5% alcoholic potassium hydroxide. Aliquots of the hydrolysate were used for total cholesterol determination (3).

Results. Several interesting findings resulted. Regression (Table I) of accumulated (187% increase) liver cholesterol of Group B mice required a total of 4 weeks after return to the cholesterol free diet (CFD). On the other hand, animals fed CFD plus 0.5% cholic acid (Group C) showed no regression of elevated liver cholesterol within the same interval. The accumulated liver cholesterol of animals fed CFD supplemented with 0.5% hyodeoxycholic acid (Group D) regressed to near normal levels by end of first week, and dropped to 50% below normal cholesterol level by the end of 2nd week. This level was maintained through the end of 4th week. Addition of equal percentages of both bile acids (Group E) showed that at this concentration hyodeoxycholic acid reverses the inhibitory effect of cholic acid on liver cholesterol mobilization.

Data on mobilization of accumulated mouse carcass cholesterol are presented in Table II. There was a 26% increase in carcass cholesterol during the 3-week build-up period. Regardless of dietary supplement, carcass cholesterol levels returned to normal by the end of 2nd week.

It appears that in the mouse the control of cholesterol metabolism in extrahepatic tissues —with exception of blood(4)—is independent of the effects of bile acid and of liver *per se.*

The mechanisms by which bile acids influence liver cholesterol mobilization remain doubtful. Cholic acid may inhibit cholesterol mobilization (a) by preventing excretion of α and β sterols, or (b) by blocking oxidation of cholesterol to bile acids obligatory for secretion(5,6). Hyodeoxycholic acid may have the opposite effects: that is, it may promote rapid excretion of α and β sterols, or may increase the rate of cholesterol oxidation. The reversal

TABLE II. Mobilization of Accumulated Carcass Cholesterol in Mice Fed Cholic and Hyodeoxycholic Acids, as a Function of Time.

Group	Carcass* total cholesterol, mg/g carcass				
	0 time	1 wk	2 wk	3 wk	4 wk
A. Normal—CFD†	2.45 ± .38	2.43 ± .19	2.66 ± .29	2.55 ± .36	2.55 ± .37
B. Regression—CFD	3.08 ± .43	3.05 ± .16	2.60 ± .19	2.48 ± .29	2.33 ± .49
D. *Idem* + 0.5% hyodeoxycholic acid	3.08 ± .43	3.11 ± .29	2.76 ± .15	2.68 ± .21	2.62 ± .20
E. " + 0.5% cholic acid + 0.5% hyodeoxycholic acid	3.08 ± .43	3.09 ± .14	2.68 ± .19	2.56 ± .37	2.78 ± .35

Statistical comparison of values:

Groups	0 time	1 wk	2 wk	3 wk	4 wk
A and B	$P < .01$	$P < .01$	$P = .64$	$P = .69$	$P = .32$
A and D	"	"	$P = .42$	$P = .45$	$P = .69$
A and E	"	"	$P = .90$	$P \cong 1.00$	$P = .24$

* without liver. † CFD = cholesterol-free diet. ± numbers are stand. dev.

of cholic acid effects by hyodeoxycholic acid suggests that the latter substance acts as an antimetabolite of cholic acid. Although general increases or decreases in metabolic rate would have similar effects on cholesterol mobilization, we have shown(4,7) that under our experimental conditions, the metabolic rate remains constant.

It was hoped that the effects of hyodeoxycholic acid in the mouse could be duplicated in other species, but in preliminary studies in the rat, this substance was ineffective at equivalent dosage levels.

Summary. The effects of cholic and hyodeoxycholic acids on mouse liver and carcass cholesterol mobilization were investigated. Cholic acid inhibited liver cholesterol mobilization, while hyodeoxycholic acid promoted a rapid elimination of accumulated liver cholesterol. Hyodeoxycholic acid reversed the inhibition caused by cholic acid. Neither of these substances effected the rate of carcass cholesterol mobilization.

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Aliphatic Amino Compounds as Inhibitors of Plasminogen Activation.

(25585)

ALBERT SJOERDSMA* AND INGA MARIE NILSSON
Medical Clinic, General Hospital, University of Lund, Malmö, Sweden

The fibrinolytic system of man is the subject of increasing interest(1). While activators such as streptokinase have been studied extensively, inhibiting agents have received relatively little attention. ε-amino-n-caproic acid (ε-ACA) was first mentioned in this connection in 1957 by Japanese workers who described it as an anti-plasmin agent(2). It was shown subsequently by Ablondi *et al.*(3)

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* Visiting Scientist, N. H. Inst., Bethesda, Md.

and Alkjaersig *et al.*(4) that the primary action of this compound is to inhibit plasminogen activation. It is of interest that Müllertz (5) had reported earlier that lysine and ornithine are also inhibitors of plasminogen activation. A systematic study of aliphatic amino compounds seemed desirable. An *in vitro* system for studying plasminogen activation was utilized for this purpose.

Materials and methods. Amino compounds. The compounds tested were obtained from commercial sources. They were amino acids and diamines with a carbon chain of 3 to 8 C's: 1) 3 C's: β-alanine, 2) 4 C's: *DL-*α, *DL-*β and γ-aminobutyric acid and putrescine · 2 HCl, 3) 5 C's: Δ-aminovaleric acid · HCl, Δ-aminolevulinic acid · HCl (methyl ester), *L*-glutamic acid, ornithine · 2 HCl, (*L*+)-arginine · HCl and cadaverine · 2 HCl, 4) 6 C's: ϵ-aminocaproic acid (ϵ-ACA), *DL*-norleucine and *L*-lysine · HCl, and 5) 8 C's: ω-aminocaprylic acid. The ϵ-ACA used here was a commercial preparation from L. Light & Co. Ltd., Colnbrook, England. On high voltage electrophoresis it was homogeneous. *Constituents of in vitro systems*: Bovine fibrinogen with a coagulability of 97-98% was prepared according to Blombäck and Blombäck(6). The thrombin used was thrombin Roche (Topostasin). A preparation of human plasminogen + proactivator was obtained from Kabi, Stockholm. It had been prepared by Kline's modification of Christensen and Smith's procedure from human plasma Fraction III and contained 0.2 casein unit/mg. Human plasmin was a preparation of spontaneously activated human plasminogen with 0.2 casein unit/mg. A highly purified streptokinase preparation containing 500 units/μg nitrogen was supplied also by Kabi, Stockholm. *System for testing inhibition of plasminogen activation.* The inhibitory effect of ϵ-ACA and related substances on plasminogen activation by streptokinase was tested in a clot system at pH 7.4 and 37°C. Each tube contained the following components in their order of addition: 0.1 ml plasminogen solution (0.1 mg/ml 0.9% NaCl) + 0.5 ml Tris buffer of pH 7.4 and ionic strength 0.15 containing a sufficient amount of the compound to be tested to give final concentrations shown in Table I + 0.2 ml of 0.5% bovine fibrinogen in Tris buffer + 0.1 ml streptokinase solution (50 units/ml 0.9% NaCl) + 0.1 ml thrombin solution (20 N.I.H. units/ml 0.9% NaCl). Lysis time was determined from the time streptokinase was added. In absence of inhibitors an average lysis time of 10 minutes was obtained. In absence of inhibitors and streptokinase no dissolution of the clot was obtained within 48 hours. *Anti-plasmin assay.* The anti-plasmin activity of the compounds was assayed by incubation with spontaneously activated human plasminogen and determining degree of inhibition produced. Plasmin aliquots (0.4 ml containing 1.6 casein units) were incubated with 0.1 ml of a 10^{-1} M solution of the compound for 10 minutes at 37°C. Each sample was then assayed for residual plasmin on heated fibrin plates (subjected to 85°C for 45 minutes) as described by Nilsson and Swedberg(7). The result was expressed as percentage inhibition of the original plasmin solution.

Results. Activity of the various compounds, in order of potency as inhibitors of plasminogen activation, is shown in Table I. ϵ-ACA, the reference compound, was most potent. Only one other substance, Δ-aminovaleric acid, showed activity at a concentration of 10^{-3} M. Δ-aminolevulinic acid and ω-aminocaprylic acid had significant activity at 5×10^{-3} M. γ-aminobutyric acid and lysine at 10^{-2} M and putrescine, cadaverine and ornithine only at 5×10^{-2} M. Even at the latter concentration, no inhibitory activity was found with the other compounds tested.

None of the substances were anti-plasmin agents as measured by the procedure described under methods.

Discussion. The results indicate that aliphatic amino acids with a carbon chain length of 4 to 8 are inhibitors of plasminogen activation, maximal activity residing in the 6-carbon compound, ϵ-ACA. That the terminal position for the amino group is critical is shown by the fact that whereas γ-aminobutyric acid has significant activity, α and β-aminobutyric acid do not. Furthermore, ϵ-ACA is a potent inhibitor whereas α-aminocaproic acid (norleucine) is inactive. A function for the carboxyl

TABLE I. Inhibitors of Plasminogen Activation *In Vitro*.

	Concentration and lysis times*				
Inhibitor	5×10^{-2}M†	10^{-2}M	5×10^{-3}M	10^{-3}M	10^{-4}M
ε-Aminocaproic acid		>20 hr	ca 4 hr	44	16
Δ-Aminovaleric "	>20 hr	ca 3 hr		27	15
Δ-Aminolevulinic "		ca 4 hr	52	16	11
ω-Aminocaprylic "	>20 hr	55	24	11	12
γ-Aminobutyric "	81	25		12	10
Lysine	80	24		13	12
Putrescine	45	12		10	9
Cadaverine	36	11		10	10
Ornithine	34	16		10	9

* Unlabelled values represent clot lysis times in min.; each value represents avg of at least 4 determinations in duplicate. Avg of 52 control determinations was 10 min. with range of 7-16 min.

† No prolongation of lysis time was found at 5×10^{-2}M with the following: β-alanine, α- and β-aminobutyric acid, glutamic acid, arginine, and norleucine.

group as well is shown by the fact that addition of an amino group in the α-positions of ε-ACA (α, ε-diaminocaproic acid or lysine) and Δ-aminovaleric acid (α, Δ-diaminovaleric acid or ornithine) results in considerable reduction of activity. Also, replacement of the carboxyl groups of the 4 and 5 carbon amino acids with amino groups (the diamines, putrescine and cadaverine) results in lessened activity.

ε-ACA appears to be a promising agent for clinical trial in fibrinolysis associated with various diseases or with use of streptokinase for treatment of thromboembolic episodes. Preliminary clinical studies in this laboratory confirm this impression. In addition to possible use of such compounds as therapeutic agents, the question arises whether any of them may play a role in physiologic or pathologic fibrinolysis and thrombosis. The 2 most potent substances, ε-ACA and Δ-aminovaleric acid have not been found in animal tissues. However, the latter compound has been reported by Fosdick and Piez(8) to be present in saliva of patients with periodontitis, presumably as a product of bacterial putrefaction. Local production of this inhibitor of fibrinolysis might account in part for the indolent characteristics of this disease. It is unlikely that naturally-occurring compounds such as Δ-aminolevulinic acid and γ-aminobutyric acid occur in a sufficient concentration in any tissue to exert an inhibiting effect.

Conclusions. Several aliphatic amino compounds are inhibitors of activation of plasminogen by streptokinase *in vitro*. The most potent of these are amino acids with a carbon chain length of 4 to 8 and with the amino group in the omega position. The significance of these findings is discussed.

The technical assistance of Gunilla Martinsson is appreciated.

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Mammogenesis in Ovary-Thyro-Parathyroidectomized Rats.* (25586)

R. von Berswordt-Wallrabe† and C. W. Turner
(With technical assistance of Mary E. Powell)
Dept. of Dairy Husbandry, College of Agriculture, University of Missouri, Columbia

Little is known concerning role of parathyroid glands in mammary gland growth. In our studies concerning the role of various hormones on mammogenesis, levels of estradiol benzoate (EB) and progesterone (P) were indicated which stimulated lobule-alveolar growth (as measured by total DNA) equal to that observed at the end of pregnancy, when given daily for 19 days to ovariectomized (OE) rats(1). The present experiment was undertaken to determine the effects of absence of parathyroid hormone upon mammogenesis.

Materials and methods. Sprague-Dawley-Rolfsmeyer rats were OE on day 1 of experiment and thyro-parathyroidectomized (TPE) on day 7. One group was injected daily with 2 μg EB plus 6 mg P, a second group received additional 3 μg l-thyroxine (T4)/100 g body weight (BW) from day 15 through day 33. Animals were sacrificed on day 34, 24 hours after 19th injection. Control animals were operated in the same way but not injected with hormones. All animals were injected with 1 μc I^{131} on day 31 or 32. Forty-eight hours later I^{131} uptake was checked in 3 regions: neck, body (abdomen) and bladder. Animals which showed a higher uptake in the neck region than in the body were excluded from the experiment under the assumption that removal of thyroid tissue was incomplete. Animals were killed with ether, pituitaries and the 6 posterior mammary glands removed rapidly. Processing of mammary tissue was conducted as previously described(1,2). Lobule-alveolar development was checked in whole mounts of glands.

Results. The OTPE group at levels of 2 μg EB plus 6 mg P showed mammary development (as measured by total DNA content of the 6 abdominal mammary glands/100 g body weight (BW)) equal to that of OE group. Due to significantly reduced amount of dry, fat-free tissue (DFFT)/100 g BW there was no further increase of total DNA when 3 μg T4/100 g BW were given to replace the thyroid gland, although DNA/mg DFFT was significantly increased.

Wet weight of pituitaries/100 g BW increased markedly under the influence of ovarian hormones. T4 reduced this hyperplasia but increase was still significant in relation to controls.

Completeness of thyroidectomy was shown by minimal I^{131} uptake at 48 hours. Completeness of parathyroidectomy was not determined.

Mammary glands of control animals showed duct development, whereas rats receiving ovarian hormones exhibited lobule-alveolar development. The fatty pads in which mammary glands grow were well developed in all groups except those given T4.

Discussion. Data presented are first quantitative evidence that complete mammogenesis can be induced experimentally with adequate levels of ovarian hormones in OTPE rats, confirming previous studies based on qualitative data(3). However, this observation was unexpected since T4 deficiency was expected to depress mammary gland growth. These data suggest the possibility of higher levels of ovarian hormones replacing thyroid and parathyroid hormone deficiencies. It was shown that 3 μg T4/100 g BW increased total DNA of mammary glands of OE rats when given in addition to same amounts of ovarian hormones (2). Furthermore, in pregnant rats, DNA was increased when 2.5 and 3.5 μg T4/100 g BW were injected daily. (Unpublished data).

In the present experiment, however, addition of 3 μg T4 did not increase total DNA of mammary glands of OTPE rats given ovarian hormones compared to OE rats. Since the

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† Research Fellow, Dept. of Animal Physiology and Animal Nutrition, Univ. of Goettingen, West Germany, and Medical Fellow of Population Council.

difference in the groups is a lack of parathyroid hormone, a need of parathormone under these conditions is indicated.

One observation is noteworthy in regard to effect of 3 μg T4, namely a reduction in DFFT /100 g BW associated with an increase in DNA μg/mg DFFT, in comparison with control animals. While this level of T4 did not affect BW adversely, it might influence mammogenesis adversely reducing DFFT. Lower levels of T4 may restore DFFT to normal.

Summary. Daily administration of 2 μg estradiol benzoate plus 6 mg progesterone to mature ovary-thyro-parathyroidectomized rats for 19 days resulted in mammary gland growth (DNA) comparable to ovariectomized rats receiving same treatment. Replacement therapy with 3 μg thyroxine/100 g BW depressed total DFFT and increased DNA concentration but failed to increase total DNA equal to that of ovariectomized rats so treated. These data suggest that higher levels of ovarian hormones may offset thyro-parathyroid hormone deficiencies. With 3 μg thyroxine/100 g BW total DNA of glands did not equal those of ovariectomized group. It is suggested that lack of comparable response may be due either to lack of parathormone or slight excess of thyroxine.

TABLE I. Experimental Mammogenesis in Ovary-Thyro-Parathyroidectomized Rat.

			Body wt			Pituitary		6 posterior mammary glands				
								DFFT			DNA	
Daily treatment from Day 15-33		No. of rats	Day 1	Day 34	Gain, %	Wet wt, 100 g BW	48 hr uptake of I^{131}, neck, %	Total	Total, 100 g BW	μg DNA, mg DFFT	Total	Total, 100 g BW
			g	g		mg		mg	mg	μg	mg	mg
Controls*	OE	15	263	274				359	131	23	8.3	3.1 ±.1
	OTPE	17	214	240	13.5 ±2.4	5.3 ±.2^{1}	1.3 ±.1 ^{4}	376	153 ± 9^{7}	22 ± 1^{10}	8.0	3.2 ±.1^{13}
2 μg EB + 6 mg P*	OE	12	270	272				593	218	37	22.1	8.1 ±.4^{14}
	OTPE	37	228	257	12.6 ± .8	7.7 ±.2^{2}	1.1 ±.1 ^{5}	589	229 ± 7^{8}	36 ± 1^{11}	21.0	8.1 ±.2^{15}
Idem + 3 μg T4/100 g BW*	OE	35	254	257				663	258	38	25.5	9.9 ±.3^{16}
	OTPE	33	228	254	11.0 ± .8	6.2 ±.2^{3}	.2 ±.03^{6}	518	204 ± 5^{9}	41 ± 1^{12}	21.0	8.3 ±.2^{17}

* Data from (2). BW = Body wt. DFFT = Dry, fat-free tissue. EB = Estradiol benzoate. OE = Ovariectomized. OTPE = Ovary-thyro-parathyroidectomized. P = Progesterone. T4 = Thyroxine.

Student's "t" Probability

1–2 2–3 4–6 5–6 7–8, 9	<.001
10–11, 12 11–12 13–15, 17	<.001
1–3 14–16	.001 < p < .005
8–9	.005 < p < .01
15–17	.4 < p < .2

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Concentration of ACTH in Cavernous Sinus and Peripheral Blood Collected from Unanesthetized Sheep.* (25587)

L. Z. McFarland, M. T. Clegg and W. F. Ganong (Introduced by H. H. Cole)
University of California, Depts. of Vet. Medicine and of Animal Husbandry, Davis, and Dept. of Physiology, San Francisco

Anatomical location of the pituitary and abrupt termination of its effluent veins into the cavernous sinus makes direct collection of venous blood from this gland impossible. Ganong and Hume(1) reasoned that since effluent veins of the pituitary emptied directly into the cavernous sinus in the dog, samples of blood from this site should contain high concentrations of pituitary hormones. They used a stereotaxic instrument to position a needle within the cavernous sinus and demonstrated that blood collected from this site contained a significantly higher concentration of ACTH than peripheral arterial blood. Sheep, another species in which pituitary veins empty into the cavernous sinus(2), would be an ideal experimental animal. Their docility permits easy manual restraint, and their large size is conducive to removal of a considerable volume of blood. This paper describes a simple method of puncturing the cavernous sinus in the unanesthetized sheep and presents evidence that concentration of ACTH is greater in cavernous sinus blood than in peripheral blood.

Methods. Vinylite corrosion casts of 4 sheep heads were examined to determine extent of the cavernous sinus and its connections. The cavernous sinus is a large, paired structure lying on the basisphenoid. An extensive rete mirabile cerebri is embedded within it. Effluent veins of the pituitary and ophthalmic veins enter the sinus. A small intercavernous sinus is located anteriorly and a large intercavernous sinus posteriorly. Large radicles pass from the cavernous sinus out through the foramen lacerum to connect with the internal maxillary vein of the systemic venous system. Small radicles connect with the basilar sinus, and no emissary veins are present. The cavernous sinus extends posteriorly beyond the foramen ovale, which is very large in sheep. The large size of this foramen suggested an approach to the cavernous sinus from the exterior. More than 20 sheep were used to verify that the cavernous sinus could be punctured through this foramen and that blood could be collected. Sheep were manually restrained in either a standing or a lateral recumbent position. A 4 inch by 15 or 16 gauge hypodermic needle attached to 50 ml syringe was inserted through the skin in the parotid region approximately 2 inches below the external auditory meatus and just caudal to the posterior border of the ramus of the mandible (Fig. 1). The needle was gently inserted toward the opposite eye in an anterodorsal direction. When the needle entered the foramen ovale, its penetration of the mandibular division of the trigeminal nerve caused the animal to struggle. After the needle had passed through the outer layer of the dura mater which covers the cavernous sinus, tissue resistance was no longer encountered and blood from the sinus entered the syringe. Four rams were used to verify that blood from this source contained a higher concentration of ACTH than from a peripheral source. To stimulate ACTH release, the rams were subjected for 1 minute to intermittent electrical shocks applied 5 minutes before collecting the blood. Fifty milliliters of blood from the cavernous sinus and from the recurrent tarsal vein were collected simultaneously. These samples were placed in heparinized tubes and centrifuged 5 minutes. The plasma was decanted into glass containers and frozen immediately on dry ice. Plasma ACTH concentrations were determined according to the method of Nelson and Hume(3).

The *results* (Table I) indicate that plasma samples from the cavernous sinus consistently show a higher concentration of ACTH than plasma samples from the peripheral venous source.

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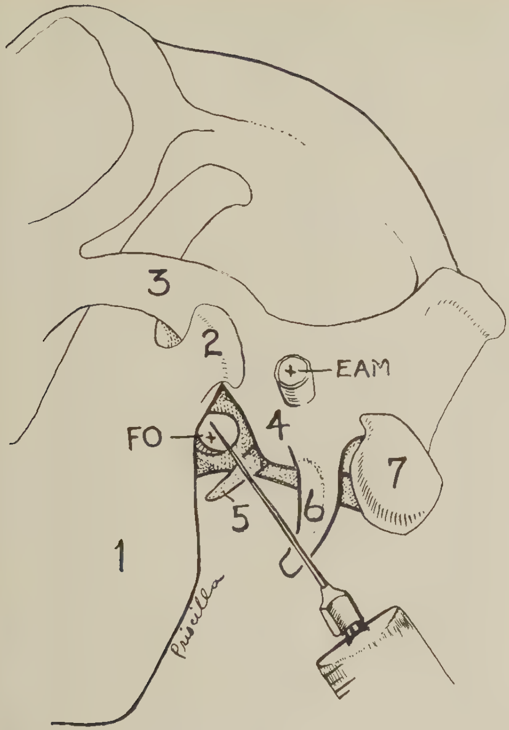

FIG. 1. Anatomical illustration of sheep skull showing site of puncturing the cavernous sinus (greater cornu of hyoid bone is removed). 1—ramus of mandible; 2—condyle of mandible; 3—zygomatic arch; 4—temporal bone; 5—muscular process; 6—paramastoid process; 7—occipital condyle; EAM—external auditory meatus, and FO—foramen ovale.

TABLE I. Concentration of ACTH in Samples of Blood Withdrawn Simultaneously from Cavernous Sinus and from Recurrent Tarsal Vein in 4 Rams.

ACTH content, milliunits/100 ml plasma	
Cavernous sinus	Recurrent tarsal vein
54	0.6
60	14
78	34
123	59

Concentration of ACTH ranged from 54 to 123 milliunits % in sinus plasma compared to 0.6 to 59 milliunits % in peripheral plasma. In any individual animal the difference between the 2 samples was greater than 2-fold. These results confirm those of Ganong and Hume(1) that pituitary ACTH is present in its highest concentration in cavernous sinus blood.

Some precautions in the use of this technic should be observed. Some dilution of cavernous sinus sample may occur if the rete mirabile cerebri is ruptured. Arterial contamination is indicated by increased redness of blood entering the syringe. This seldom occurred. If the needle is inserted too far it may damage the pituitary or penetrate the diaphragm sellae and enter the brain. The proper depth is reached when blood freely enters the syringe. If the needle is directed too far ventral, it may damage the external maxillary vein or artery causing hemorrhage. This is not serious. Following removal of blood and release of animal, no ill-effects were observed in spite of repeated samplings and sampling from either side.

Thus far blood obtained from the cavernous sinus has been examined for ACTH concentration only. It is probable that high concentrations of other pituitary hormones are also present. Studies on concentration of gonadotrophins are now in progress.

Summary. A simple technic for puncturing the cavernous sinus of unanesthetized sheep through the foramen ovale has been described. Cavernous sinus plasma showed at least a 2-fold greater concentration of ACTH than peripheral plasma collected simultaneously from the recurrent tarsal vein.

The authors are indebted to Priscilla Tippett for illustration and to Angela Boryczka, Roy Shackleford, and Vina Sera for technical assistance.

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Thermal-Resistant Populations of Foot-and-Mouth Disease Virus. (25588)

H. L. Bachrach, R. E. Patty and R. A. Pledger
Plum Island Animal Disease Lab., U. S. Dept. of Agriculture, Greenport, L. I., N. Y.

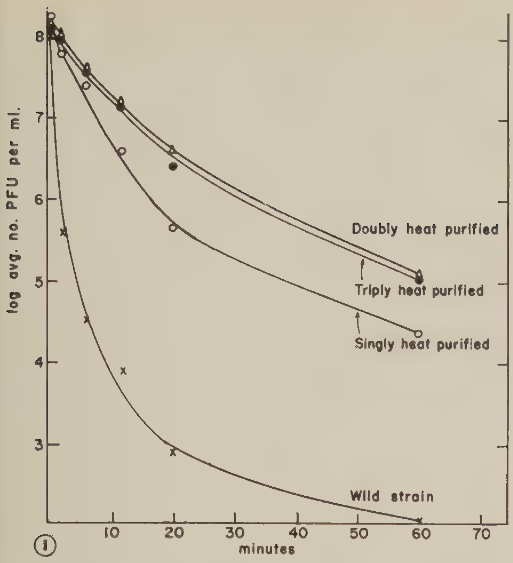
Bachrach *et al.* reported thermal resistance of a small fraction (0.001) of a wild strain of foot-and-mouth disease virus (FMDV) grown in tissue culture(1). The present communication deals with the genetic homogeneity of this strain. The study shows that the thermal-resistant virus fraction, after subsequent passages in tissue culture under continuing heat selection, yields virus populations containing a much higher proportion of thermal resistant particles than were present in the wild population. However, further passages of such populations, made without selecting out the thermal-resistant fraction, yield virus populations which have partially reverted toward the heat sensitivity of the wild strain.

Material and methods. Wild virus was FMDV, type A, strain 119, (FMDV-A119) passaged 76 times in calf-kidney cultures as previously reported(2). Infectious fluids were harvested, pooled, and stored at –40°C. As needed frozen virus was thawed to 4°C and centrifuged at 6,400 x g for 30 minutes to remove cell debris. *Cultures* as confluent sheets of cells were produced in 4-oz prescription bottles and Roux flasks from trypsin-dispersed fragments of calf kidney. *Virus infectivity measurements* were made by plaque assay as described elsewhere(2). One-tenth-ml volumes of properly diluted virus were plated in duplicate onto calf-kidney cultures in prescription bottles. After 90 minutes incubation at 37°C, the cultures were overlaid with agar medium. Plaques were read at 72 hours. A few virus titrations were also made in guinea pig foot pads and in cattle tongues. *A thermal inactivation procedure* was used which changed the temperature of the virus quickly. That is, one part of buffered virus at 20°C was mixed with 9 parts of veronal-acetate buffer held in a water bath at one-half degree higher than the temperature desired. A nearly instantaneous adjustment of temperature was thereby effected. After the desired interval of exposure, the thermal reaction was stopped by diluting the heated virus sample with an equal volume of ice-cold buffer.

Results. As previously reported, wild virus populations heated at 55°C yield, within a few minutes, a residual fraction amounting to 10^{-3} of the original virus. This fraction is much more resistant to heat than the bulk population. A similar relationship with a surviving fraction of only 10^{-5} is given in Fig. 1. The 60-min. survivors were then grown out to a high population in tissue culture and the new population was tested for its heat resistance. Fig. 1 shows that the new population (single heat purification) contains a much higher surviving fraction, *i.e.*, approximately 10^{-2}, of heat-resistant virus particles than the wild parent. Survivors of this second heat treatment were again grown to a high population and again heated. This process was repeated a third time with results presented in Fig. 1. Populations from doubly and triply heat-purified viruses appear more stable to heat than progeny from the heat residuum of wild virus. Furthermore, their curves, which approach straight lines, indicate that at least 10% of the virus particles are of the heat-resistant kind.

Differences in heat stability of wild parent virus and of progeny obtained after heat purification were thought possibly due to a sensitization of the wild parent during its freezing and thawing—a process to which progeny were not subjected. Therefore, wild parent virus and its progeny obtained after heat purification were each examined as to rate of thermal inactivation with and without prior freezing. The respective curves were identical to those in Fig. 1, indicating that freezing and thawing of heat-sensitive or heat-resistant populations have no effect on their inactivation at 55°C.

Experiments were next carried out to determine if populations with increased thermal resistance could be maintained without heating for selection. The triply heat-purified virus (Fig. 1) was subcultured several times without heat treatment and then tested for



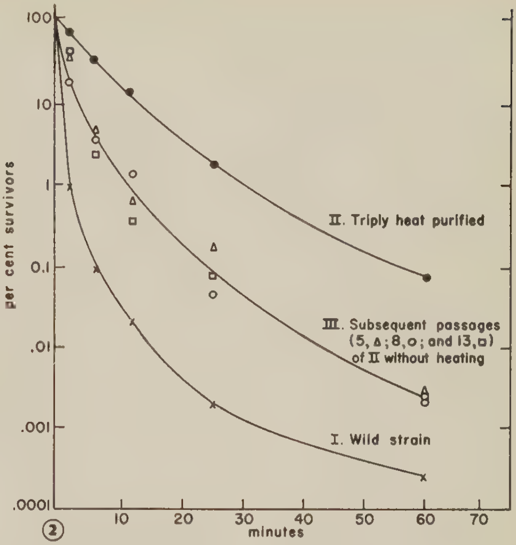


FIG. 1. Inactivation curves at 55°C for wild foot-and-mouth disease virus and of virus derived from it by single, double, and triple heat purifications and by passages in calf-kidney cultures.

FIG. 2. Inactivation curves at 55°C for wild foot-and-mouth disease virus and of various heat-resistant subcultures derived from it.

thermal resistance. After 5 such passages, (Fig. 2) partial reversion toward the heat sensitivity of the wild strain occurred with 90% inactivation at 55°C taking place 3 times faster. Further passages of virus in culture, at least through the eighth and thirteenth, caused no further change in heat resistance.

No additional differences between wild and thermal-resistant populations were detected. There were no changes in size of plaques or in their rates of development at 37°C. Growth curves in calf-kidney cultures of 2 heat-resistant populations were compared with that of the wild virus (Table I). Peak titers appeared to be reached 2.5 hours earlier when inoculated with thermal-resistant virus and there was a slightly higher liberation of progeny per cell. This was not unexpected because greater thermal inactivation during culturing would tend to spread out the logarithmic growth phase of the thermal-sensitive strain and to decrease its peak titer. Also, no significant differences in infectiousness have been demonstrated between the two kinds of populations in any of the hosts tested, *i.e.*,

TABLE I. Wild and Heat-Resistant Populations of Foot-and-Mouth Disease Virus: Growth and Comparative Infectivity.

			Population	
		Wild virus	No. 1*	No. 2†
Growth:	Time for 1 log increase in titer (min.)‡	42	35	35
	Peak titer (PFU/ml)	5.7×10^7	6.2×10^7	6.2×10^7
	Time of peak titer (hr)	15	12.5	12.5
	Avg yield (PFU/cell)	102	118	113
Infectivity tests in:	Calf-kidney culture (log PFU/ml)	8.2	7.8	8.0
	Guinea pigs (log ID_{50}/ml)	4.9	5.7	5.2
	Cattle (" ")	6.7	5.7	6.5

PFU—Plaque forming units.

* Inoculum for this population derived from triply heat-purified virus (II in Fig. 2) subcultured 14 times without heating.

† Inoculum for this population derived from triply heat-purified virus (II in Fig. 2) subcultured 13 times without heating followed by one passage after heating.

‡ During logarithmic growth phase.

calf-kidney cultures, guinea pigs, and cattle (Table I).

Discussion. Since it is assumed that suspensions of genetically reproducible units are variable in a number of characteristics, it is not surprising to find that FMDV is variable in its sensitivity to heat. The application of heat to the wild strain of FMDV appears to destroy primarily the heat-sensitive particles. Thus, by the process of selection, heat-resistant particles remain. However, in the competitive reproduction of both, heat-sensitive particles appear to have a selective advantage in their replication for they tend to predominate in progeny. If this reversion process were to continue indefinitely in absence of heat treatment, it is expected that a complete reversion to the level of heat sensitivity of the wild type would be effected. Since the isolated resistant fraction—after 13 passages without heat—retains a significant resistance to the selecting agent, the heat treatment may effect a physical change within the virus particle itself. From work being reported elsewhere(Bachrach,3) it is clear that heating at 55°C denatures an infection-initiating property of the protein coat of FMDV without appreciable effect on its ribonucleic acid core.

Summary. A small thermal-resistant fraction (10^{-3} to 10^{-5} at 55°C) of foot-and-mouth disease virus, type A, has been isolated from a wild population. Under continuing thermal selection, it breeds populations which contain much higher fractions, at least 10^{-1}, of thermal-resistant virus particles. Such populations revert partially, however, toward the sensitivity of the wild strain if not subjected to heat.

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Detection of Insulin-Binding Antibodies and Separation of Free and Antibody-Bound Insulin by Rapid Chemical Procedure. (25589)

ENOCH GORDIS* (Introduced by S. A. Berson)
Radioisotope Service, Vet. Admin. Hospital, Bronx, N. Y.

The presence of insulin-binding antibodies in serums of insulin-treated patients has been demonstrated by paper strip chromato-electrophoresis, starch block electrophoresis and ultracentrifugation of insulin-I^{131} plasma mixtures(1), by cellulose column chromatography (2) and by precipitation of insulin-antibody complexes with rabbit antihuman globulin serum(3). These technics are all somewhat time consuming, and are not suitable for analysis of large volumes of material with low concentrations of radioactivity. It seemed worthwhile to attempt rapid separation of free and bound antibody-insulin by chemical procedure. Bates *et al.*(4,5) showed that in 76% ethanol saturated with NaCl most plasma proteins are precipitated at room temperature, while insulin remains largely soluble. The present paper describes adaptation of a modification of this procedure to separate free insulin from antibody-bound insulin.

Materials and methods. Control (non-immune) serums were obtained from subjects who had never been treated with insulin. Antiserums were obtained from insulin-treated patients. Donors of "immune" serums were not clinically insulin-resistant and required less than 75 units of insulin daily to control their diabetes. "Resistant" serums came from donors who required at least 150 units of insulin daily at the time their blood was drawn. All "immune" and "resistant" serums had been studied in detail previously(6). Insulin-I^{131} (specific activity, 38 - 73 mc I^{131}/mg insulin) and gamma globulin-I^{131} were prepared from crystalline beef insulin[†] and Cohn fraction II[‡] respectively, by methods described previously (7). Human serum albumin-I^{131} was purchased from Squibb Labs. Paper-electrophoresis and hydrodynamic-flow chromatography were performed according to published methods(1). During preparation of insulin-I^{131} at high specific activity some labeled insulin undergoes radiation damage such as reductive splitting of the disulfide bonds(8) and binds non-specifically to plasma proteins, primarily to alpha-globulins(1). Results given below are corrected for damaged insulin.[§] I^{131}-labeled proteins were incubated with serum at 37°C for 4 hours, then refrigerated at 4°C overnight. Fifty microliters of the mixtures were added to 1 ml of the various precipitating solutions. A white flocculent precipitate formed immediately in all cases and was separated immediately by centrifugation. Radioactivities in whole mixtures and precipitates were then assayed under similar geometrical conditions in a well-type scintillation counter. At the same time, aliquots of insulin-serum mixtures were analyzed for bound and free insulin by paper chromato-electrophoresis as described previously(1).

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* Fellow of Dazian Fn., 1958-1959.

Results. NaCl-saturated 76% ethanol solution was in itself not suitable for separation of free and bound insulin at room temperature. Although more than half of albumin-I^{131} and almost all of gamma globulin-I^{131} were precipitated while 85% of insulin-I^{131} remained in solution, only 48% of antibody-bound insulin was precipitated (Table I).

TABLE I. Precipitable Radioactivity in NaCl-saturated 76% Ethanol at 25°C pH 6.4.

	Control serum	Insulin-resistant serum[*]
Albumin-I^{131}	58%	
Gamma-globulin-I^{131}	94%	
Insulin-I^{131}	15%	48%

[*] 90% of insulin-I^{131} was bound to antibody in this serum as determined by paper electrophoresis.

Temperature and pH were found to influence considerably the solubility of free and antibody-bound insulin. In general, lower temperatures favored precipitability of insulin-antibody complexes more than that of free insulin whereas increasing acidity increased precipitation of free insulin (Table II). Accordingly, the best separations were obtained at high pH and low temperature. At –10°C and pH 6.6 - 8.0, over 90% of the antibody-bound insulin could be precipitated, but as much as 25% of free insulin-I^{131} was precipitated as well (Table II). On increasing the pH to 10.2 or above, precipitability of free insulin was decreased considerably without significant loss in precipitability of insulin-antibody complexes.

The solution found to achieve the best separation was salt-saturated 76% ethanol in 0.01 M KOH. Following precipitation at –12°C to –10°C, (achieved in an ice-brine mixture) centrifugation was performed at 0°C since the small amounts of free insulin which coprecipitate at the lower temperature redissolve rapidly while insulin-antibody complexes remain as well-packed precipitates. Results of this procedure applied to the serums of 23 control subjects and insulin-treated patients are summarized in Table III. Precipitation of insulin-I^{131} in each case was compared with extent of insulin binding by antibody on

TABLE II. Precipitable Insulin-I^{131} in NaCl-saturated 76% Ethanol Solution.[*]

	0°C			–10°C
	pH 8[†]	pH 6.6[‡]	pH 3.5[§]	pH 8[†]
% insulin-I^{131} precipitated from control serum	10-11%	14-16%	27-31%	15-25%
% antibody-bound insulin precipitated from resistant serum	78-80	74-76	85-87	90-95

[*] Each set of values expresses range of 4 replicate determinations.

[†] .01 M borate buffer.

[‡] " phosphate " .

[§] " acetate " .

[†] The Laboratory is indebted to Doctors Otto Behrens and C. W. Pettinga of Eli Lilly Research Labs. for generous supplies of crystalline insulin.

[‡] Prepared by Dr. Eric Pollaczek.

[§] This damage generally amounted to about 10-15% of total radioactivity as determined by paper chromato-electrophoresis of insulin-I^{131} in control serum. In antiserums, damaged fractions which bind to α and β-globulins and to albumin are readily distinguished from antibody-bound insulin which migrates at front of gamma-globulin zone(1).

TABLE III. Precipitation of Insulin-I^{131} from Incubated Mixtures of Insulin-I^{131} and Serum by NaCl-saturated 76% Ethanol Solution in 0.01 M KOH at −12°C.

Serum dilution	Type	% radioactivity precipitated	% radioactivity bound to Ab by chromato-electrophoresis	% Ab-bound insulin precipitated
Undil.	Control	7	None	
		15	"	
		6	"	
		6	"	
		6	"	
	Immune	51	82	62
		75	86	87
		63	71	89
		55	85	65
		90	91	90
		57	89	64
		75	75	100
		96	95	101
		89	90	99
		87	90	97
		85	90	95
		76	84	90
1:10	Resistant	86	90	91
"		70	79	89
"		87	90	96
1:50		78	90	87
1:10		91	90	101
1:50		80	80	100

paper chromato-electrophoresis. No more than 15% of free insulin was precipitated from control serums, while in 15 of 18 antiserums over 87% of the bound insulin was precipitated.

A large number of experiments was performed in which salt and ethanol concentrations were varied. In general, decrease in salt concentration or increase in alcohol concentration favored precipitation of free insulin with the serum proteins With the modifications of temperature and pH described, the medium of Bates *et al.*(4,5) was found to be the most effective for the present purpose.

Summary. A rapid chemical method for detection of insulin-binding antibodies and separation of free and antibody-bound insulin in human antiserums is described. With NaCl-saturated 76% ethanol solution in 0.01 M KOH at −12°C to −10°C, 87% - 100% of antibody-bound insulin was precipitated from 15 of 18 insulin antiserums whereas 85% - 94% of unbound insulin in control serums remained in solution.

The author is indebted to Drs. S. A. Berson and R S. Yalow for aid and encouragement and for supplying insulin-I^{131} and serums. Thanks are also due Frieda Steiner for secretarial assistance.

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Effect of Phentolamine (Regitine®) on Human Plasma FFA *in vivo*.*

(25590)

ROBERT F. KLEIN AND MORTON D. BOGDONOFF
Dept. of Medicine, Duke University Medical Center, Durham, N. C.

Studies of plasma free fatty acid (FFA) levels in man and animals suggested that the sympathetic division of autonomic nervous system affects lipid metabolism(1). Administration of noradrenalin and adrenalin causes rapid rises in plasma FFA levels. Administration of an autonomic ganglion blocking

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TABLE I. Individual Response to Phentolamine Infusion FFA (μm/l) in 9 Subjects.

Dose, mg	Resting	During saline infusion	During phentolamine infusion	Time after end of phentolamine infusion, min.					
				10	20	30	40	50	60
5	480	498	—*	856	785	836	840	—	1157
5	384	351	—	465	408	454	—	370	385
15	507	429	661	692	672	—	—	—	621
”	611	813	721	1032	983	878	879	908	883
30	601	651	901	1079	1070	1082	—	1187	—
”	566	543	545	752	699	701	718	700	741
”	292	287	355	654	—	935	—	617	—
† ”	754	664	1078	1158	1013	879	—	739	568
”	442	384	574	606	721	664	—	654	—

* — indicates no samples taken.

† Subject J.S. referred to in text.

agent lowers plasma FFA levels during the blockade(2). This study reports the effect of infusion of an adrenolytic agent (phentolamine, Regitine®) upon plasma FFA levels in man.

Methods. Eight hospitalized males and one volunteer male university student were studied. Their ages ranged from 19-56. All subjects were considered free of known metabolic abnormalities. None of the subjects was obese. They fasted 10-16 hours (overnight). All were previously informed as to the nature and general technic of the test. Subjects were studied in the supine position, with blood pressures and pulse rates taken by auscultation at 5-10 mintue intervals. Venous blood was drawn from a forearm or antecubital vein through an indwelling #18 Cournand needle. After a 20 minute rest period following needle placement, a saline infusion was given at 1-2 cc/min through a #18 Cournand needle in the opposite arm. By utilizing a 3-way stopcock, phentolamine infusion was started. Dosage of phentolamine was as follows: 5 mg in 2 subjects, 15 mg in 2 subjects and 30 mg in 5 subjects (the latter 2 doses given by a constant infusion pump at approximately one mg/min). Following the phentolamine infusion, blood samples were drawn at 10 minute intervals for determination of glucose (Somogyi-Nelson method) and FFA (Dole method).

Results. Table I lists FFA values obtained. Although there were no consistent changes during infusion, all subjects showed increased FFA levels following administration of phentolamine. These higher levels tended to fall after 20-30 minutes but in only one subject († in Table I) did they return to pre-injection levels at one hour after completion of infusion. Glucose values were essentially unchanged during and following phentolamine infusions.

All subjects showed a transient increase in pulse rate ranging from 4 to 26 beats/min during the first few minutes of infusion, with some subjects maintaining the increased pulse rate for duration of infusion. Blood pressure effects were as follows: A systolic and diastolic fall of 5-10 mm Hg occurred during the first 4-5 minutes of infusion, was followed by increase in systolic pressure, with diastolic pressure remaining depressed. The overall effect was an increase in pulse pressure of 10-20 mm Hg.

Discussion. The mechanism of this abrupt rise in plasma FFA levels following administration of an adrenergic blocking agent is unknown. The same phenomenon has been noted by Havel and Goldfien in the dog, using dibenamine(3). Benfey, Ledoux and Melville have demonstrated a marked increase in urinary catechol amines during development of adrenergic blockade by dibenamine(4). It is conceivable that the FFA response to phentolamine is one manifestation of a rebound transient increase in adrenergic activity.

Summary. 1) Nine human subjects were given phentolamine (Regitine®) infusions to observe the effect upon plasma FFA levels. 2) There was a consistent abrupt rise in venous FFA levels ranging from 100-500 μm/l without blood glucose changes. FFA values remained higher 1 hour after infusion. 3) The mechanism of this phentolamine effect on lipid metabolism is not known.

We thank Dr. William E. Wagner, Ciba Pharmaceutical Products, Inc., for generous supply of Regitine® used.

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Relationship Between Ribonuclease and Certain Anionic Polymers in Utilization of Mevalonic Acid by Liver Homogenates.* (25591)

LEMUEL D. WRIGHT, MARCIA LOEB AND ROSEMARIE HANSON
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Preincubation of essentially whole homogenates of rat liver with crystalline ribonuclease (RNAse) abolishes capacity of these homogenates to convert mevalonic acid (MVA) into non-saponifiable material (NSF) or cholesterol (CHL). Control homogenates similarly preincubated ordinarily retain the capacity for biosynthesis(1). Inability of RNAse-treated homogenates to utilize MVA is associated with absence of ATP in these preparations. Homogenates preincubated with RNAse and an autoclaved liver extract (ALE) previously treated with an anion exchange resin to remove residual ATP maintain a level of ATP and convert MVA to NSF and CHL(2). Evidence has been presented for existence in these heated liver extracts of a factor concerned with maintenance of a functional level of ATP in homogenates, presumably involving oxidative phosphorylation(3,4), and a tentative mechanism for explaining the experimental data has been proposed(2,5,6). Allfrey and Mirsky have previously described a possibly somewhat similar system involving isolated thymus nuclei which when pre-treated with *deoxyribonuclease* lose capacity to convert amino acids into protein or purine and pyrimidine precursors into nucleic acids. The treated nuclei are devoid of ATP but the synthetic capacity is retained on preincubation with DNA or may be established by addition of DNA after inactivation. Activity is not confined to DNA, however, and RNA as well as a number of anionic polymers substitute for DNA(7,8). It is our purpose to point out that DNA and certain anionic polymers have an effect similar to the factor of ALE in the liver homogenate system and that these anionic polymers, but not ALE, are also inhibitors of RNAse. A conclusion that all anionic polymers necessarily function in the same way in maintenance of ATP levels in tissue may not be justified.

Methods and material. The experimental procedures involved in biosynthesis of NSF and CHL from MVA by rat liver homogenates have been described(9). In brief, cooled liver from young rats is homogenized quickly in "loose" homogenizer with nicotinamide-phosphate-magnesium buffer and centrifuged at slow speed to remove connective tissue. This essentially whole homogenate is dispensed into flasks containing ATP, DPN, succinate and RNAse where indicated, as well as various materials or combinations of materials to be studied with respect to biosynthesis of NSF. The flasks are aerated with a stream of oxygen, stoppered, and preincubated with agitation. Following preincubation MVA-2-C^{14} is added to each flask, aeration is repeated and flasks reincubated. Then contents of flasks are saponified, extracted with petroleum ether, extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. The results given are in terms of cpm/incubation flask. Details of procedure are essentially as described previously. Despite use of nicotinamide as inhibitor of DPNase in the homogenizing buffer, DPN some-

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times is limiting in the system employed. For this reason it is recommended that a level of 10 mg/flask rather than 1 mg previously described be used The various polymers were studied after solution in water with neutralization or solution in buffer. The appropriate controls contained by corresponding amount of water or buffer. Heparin and DNA were commercial preparations. Several samples of polyethylene sulfonate (PES) were used including material with molecular weights of 27,600, 12,900 and 5,700. ALE was prepared by treating fresh guinea pig liver in the cold with twice its weight of water in Waring Blendor for about a minute. The brei was then autoclaved at 105 - 110° for 20 min and the mixture cooled and centrifuged at 10,000 x g for 20 min. Determinations of RNAse activity were done essentially by procedure of Kunitz (10) as modified by Zöllner and Fellig(11) which depends upon the fact that when RNAse hydrolyzes RNA there is a decrease in absorption at 300 $m\mu$. The substrate RNA solution was prepared by dissolving 25 mg commercial yeast RNA in 25 ml of 0.1M, pH 5.0, acetate buffer. The RNAse solution was prepared by dissolving 2 mg crystalline RNAse in 200 ml of water. In a typical experiment 2 cuvettes were prepared as follows: control cuvette, 1 ml RNA solution and 1.5 ml water; RNAse cuvette, 1 ml RNA solution and 0.5 ml of water. The cuvettes were placed in Beckman DU spectrophotometer and with the control cuvette in position, the instrument was arbitrarily adjusted to read an optical density of 0.175 at 300 $m\mu$. One ml of RNAse solution was then added to the RNAse cuvette and relative optical density of this cuvette determined at intervals over 10 - 15 min. Ordinarily the instrument stayed in balance but on occasion it became necessary to make minor adjustments so that the control cuvette maintained an apparent optical density of 0.175. Where various materials were studied for effect on RNAse activity they were added in 0.5 ml to each cuvette at the expense of water. Each experiment then involved a rapid series of determinations with a pair of cuvettes both containing same amounts of RNA and water or aqueous solution of material to be examined while one contained in addition 10 γ of RNAse. Suitable controls demonstrated that the RNAse solution maintained full activity during experiments. In experiment involving ALE as substrate 1 ml of this material replaced the 1 ml of RNA solution used in the other RNAse experiments.

TABLE I. Biosynthesis of NSF as Influenced by Preincubation with Various Materials.

Exp.	RNAse*	Supplement	NSF, cpm
1	—	None	6852
	+	”	1138
	+	Liver extract, 5 ml	6811
2	—	None	5368
	+	”	1445
	+	DNA, 1 mg	3498
	+	” 10	5430
	+	RNA, 1	1636
	+	” 10	3489
3	—	None	8415
	+	”	293
	—	PES, 1 mg added prior to preincubation	8461
	—	3 *Idem*	7969
	—	5 ”	7416
	—	10 ”	6861
	+	1 ”	8222
	+	3 ”	8126
	+	5 ”	7370
	+	10 ”	7185
	—	10 mg following preincubation	6938
	+	10 *Idem*	7112
4	—	None	7787
	+	”	630
	—	Heparin, 1 mg added prior to preincubation	7438
	—	5 *Idem*	7516
	—	10 ”	7782
	+	1 ”	7814
	+	5 ”	7893
	+	10 ”	7330
	—	10 mg following preincubation	6979
	+	10 *Idem*	10222

* RNAse where used was present at a level of 2.5 mg/flask.

Results. The results of a number of representative biosynthetic experiments are summarized in Table I. RNA, DNA, heparin, and PES are effective in preventing inactivation of MVA utilization, accomplished by preincubation of essentially whole homogenates with RNAse. RNA is markedly less active than the other polymers, as expected, since it is the natural substrate for the enzyme RNAse used to inactivate the system. Both heparin and PES, the 2 "unnatural" polymers studied, are as active in "reversing" RNAse inactivation, *i.e.* on addition after preincubation rather than before, as they are in preventing inactivation in the first place.

A previous paper reported that RNA is inactive in the liver homogenate system. Results of an experiment summarized in Table II afford an explanation for this apparent discrepancy. RNA and DNA were compared each at a level of 5 mg/flask against various levels of RNAse. It is apparent that at a marginal level of RNAse, RNA is active in the system while at high levels of RNAse, RNA is essentially inactive. DNA, since it is not influenced by RNAse, is equally active at all levels of the enzyme. It appears that for valid determinations of activity materials should be examined against a marginal level of RNAse.

PES, heparin, and DNA are active in that order as inhibitors of RNAse (Fig. 1). Half maximum reversal of RNAse is obtained with about 2.5 γ PES, 12.5 γ of heparin and about 50 γ per cuvette of DNA. In the latter instance complete reversal of RNAse is not obtained with any level of DNA. Since each cuvette contained 10 γ of RNAse it is apparent that inhibition ratios for one-half maximum activity vary from about 0.25 with PES to about 5 for DNA. As indicated by Exp. 4, ALE does not influence RNAse activity at a ratio of 1 ml/10 γ of RNAse. Samples of ALE examined in this assay were previously shown to be active in preventing RNAse inactivation of MVA utilization in the homogenate system. The data of Exp. 5 show that the ALE does not contain amounts of RNAse-sensitive material detectable by this procedure. RNAse is not sensitive to relatively large amounts of ATP as indicated by Exp. 6.

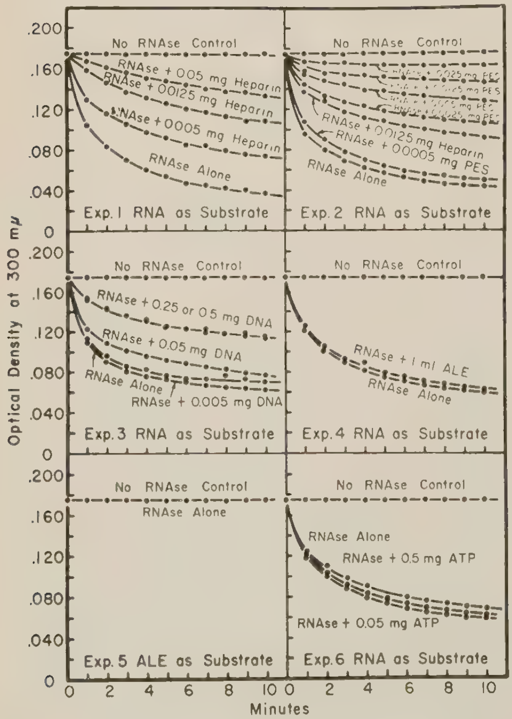

FIG. 1. Influence of various anionic polymers, ATP, or ALE on activity of RNAse.

TABLE II. Biosynthesis of NSF as Influenced by Preincubation with RNA and DNA at Various Levels of RNAse.

RNAse, mg	Supplement	NSF, cpm
0	None	5790
5.0	"	21
0	RNA, 5 mg	5482
2.5	*Idem*	465
5.0	"	31
10.0	"	24
0	DNA, 5 mg	5569
2.5	*Idem*	6634
5.0	"	6475
10.0	"	4745

Discussion. Results of biosynthetic studies demonstrate that the presence of certain polymers prevents or reverses inactivation of MVA utilization that is accomplished by preincubation with RNAse. It should be recalled that amounts of RNAse required to accomplish this inactivation are relatively large. Nevertheless in these experiments biosynthesis usually occurs when polymer to enzyme ratios of around 1 are used. In *in vitro* experiments RNAse inactivation occurred with polymer to enzyme ratios of 0.25 to 5 depending upon the polymer. As far as ALE is concerned, weight ratios are not applicable because of the crude state of the active component but 5 ml of extract ordinarily permits MVA utilization in presence of 1 - 5 mg of RNAse, a volume to weight ratio of about 1 - 5. ALE did not influence RNAse activity in the *in vitro* system at a level of 1 ml to 10 γ (0.01 mg) of RNAse, a corresponding volume to weight ratio of 100. Since anionic polymers appear to have essentially the same activity against RNAse in the 2 systems it is quite reasonable to suggest that the effects on biosynthesis may be attributable to RNAse inhibition. It will be recalled, however, that the "unnatural" polymers reverse RNAse inactivation as well as prevent it. This observation suggests, but does not necessarily prove, that they function in a positive way by fulfilling a requirement of the system rather than by inhibiting RNAse. On the other hand, ALE is inactive against RNAse *in vitro*. It is difficult to see how its effect in biosynthesis can be explained on the basis of RNAse inhibition.

Summary. Liver homogenates preincubated with RNAse, in contrast to controls, do not incorporate mevalonic acid into nonsaponifiable material or cholesterol. This inactivation by RNAse is preventable or reversible with an autoclaved extract of liver or with certain anionic polymers including polyethylene sulfonate, heparin, DNA and even RNA. The polymers also inactivate RNAse *in vitro* at comparable ratios of inhibitor to enzyme used in the biosynthetic experiments. Inactivation of RNAse *in vitro* is not observed, however, with comparatively high levels of liver extract. It is concluded that liver extract does not function in the homogenate system merely by inactivation of RNAse but this interpretation may be applicable to the anionic polymers.

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Hemagglutination-Inhibition and Serum Neutralization Response of Horses To Eastern Equine Encephalomyelitis Virus.* (25592)

FRANK M. HETRICK, FRANCES S. YANCEY, P. ARNE HANSEN AND ROBERT J. BYRNE
(Introduced by R. J. Huebner)
Depts. of Microbiology and Veterinary Science, University of Maryland, College Park

Hemagglutinating activity for Japanese B encephalitis, one of the arthropod-borne group of viruses, was first reported in 1950(1). Subsequent work revealed that other members of this group of viruses also agglutinate erythrocytes(2-5), and methods for hemagglutination-inhibition (HI) tests were developed concurrently. Information, other than from this laboratory(6), on use of the HI test for clinical and epidemiological studies of eastern equine encephalomyelitis (EEE) has been found in only one instance. Groot *et al.*(7) testing human sera for EEE found none to be positive for either HI or neutralizing antibodies. The experiments reported here were designed to compare use of HI and neutralization tests in detecting the immunological response of horses experimentally infected with EEE virus.

Materials and methods. The EEE virus used was the V-52 strain, isolated from a naturally infected horse in Maryland in 1956. The strain had undergone 17 mouse brain passages prior to inoculation of the horses. The 4 young horses used for experimental work were obtained locally from non-enzootic areas. Three horses (#126, #127, #128) were inoculated intradermally and one (#115) was inoculated subcutaneously with a virus suspension containing approximately 10^6 mouse

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TABLE I. Serological Response of Horses Experimentally Infected with EEE Virus.

	Horse 115		Horse 126		Horse 127		Horse 128	
Time post-inoc.	HI titer	Neutralization index ($\log_{10}$)	HI titer	Neutralization index ($\log_{10}$)	HI titer	Neutralization index ($\log_{10}$)	HI titer	Neutralization index ($\log_{10}$)
0	N[*]	<1	N	<1	N	<1	N	<1
5 days	40[†]	"	10	1.2	20	"	20	"
7			20	1.4	80	"	40	"
10	1280	1.4						
14	"	2.4	320	1.9	320	2.1	320	1.7
4 wk	320	3.2	40	2.8	80	2.4	80	2.2
2 mo	160	3.5	"	2.6	"	"	"	2.4
4	"	3.2	"	2.9	40	2.7	40	2.3
6	"	3.6	"	3.1	"	3.4		
9	"	3.3	20	2.7	"	3.5		
12	40	3.6			160	3.2		

[*] No inhibition at 1:10 dilution of serum.

[†] Reciprocal of highest serum dilution causing complete inhibition.

LD_{50}'s. Serum was collected at various intervals listed in Table I. Neutralization tests were performed by combining equal amounts of undiluted serum and serial 10-fold dilutions of virus. Following incubation of serum-virus mixtures for 2 hours in 37°C water bath, 3-week old Swiss mice were inoculated intracerebrally. The inoculum was 0.03 ml and 5 mice used for each serum-virus mixture. Mice were observed for 10 days, and 50% mortality endpoints were calculated by the method of Reed and Muench(8). Appropriate controls were included and results reported as the $\log_{10}$ of neutralization index. Hemagglutinin was prepared according to the method described by Chanock and Sabin for WEE(3) with following modifications. The mouse brain suspension was made in pH 9.0 borate[†] and was given a single centrifugation at 4000 rpm for 30 min at 4°C. The hemagglutination (HA) titration and HI tests were conducted according to method described by Clarke and Casals(5). Day-old chick erythrocytes were suspended in a diluent[‡] which when combined with equal amounts of pH 9.0 borate yielded a final pH of 6.3. Sera were extracted twice with acetone and the dried precipitate resuspended in pH 9.0 borate to yield an initial 1:10 dilution. Further dilutions were carried out in 2-fold steps to a dilution of 1:5120. Results are reported as the highest dilution of serum giving complete inhibition of hemagglutination.

Results. Results of serological tests on 4 experimentally infected horses are shown in Table I. None of these horses had significant EEE neutralizing or HI antibody prior to their inoculation, and clinical disease did not develop in any exposed animals. Evidence of infection was measured by appearance of HI antibody which became detectable the fifth day after inoculation in all 4 horses, whereas neutralization tests on these same sera failed to yield any significant antibody levels. It appears from Table I that significant levels of neutralizing antibody, *i.e.*, neutralization index of ≥ 50, were attained between 7th and 14th days. The HI titers of the 7-day sera available from 3 horses showed a 2-fold or greater increase over the 5-day sera, and by the 14th day had reached their maximum values. The HI titers began to decline after 14th day but were still detectable one year after inoculation, though at a low titer. The level of neutralizing antibody, on the other hand, reached its maximum level several months post-inoculation, the time varying with each horse. In contrast to the HI titers, high levels of neutralizing antibody persisted for at least one year.

Results of HI tests performed on sera from survey horses and ponies having significant neutralizing antibody levels are shown in Table II.

[†] *Boric Acid Stock Solution*: 0.2M H_3BO_3; 0.2M KCl in 0.12M NaCl. *pH 9.0 Borate*: Stock solution: 100 ml; 0.2N NaOH: 42.6 ml; qs to 400 ml with 0.12M NaCl.

[‡] To prepare 1 liter of diluent, combine 100 ml of 1.5M NaCl with 70 ml of 0.05M Na_2HPO_4, and 151.5 ml of 1.0M NaH_2PO_4, and qs to volume with demineralized water.

TABLE II. HI Test Results on Equine Sera Containing Significant EEE Neutralizing Antibody Obtained 8 or More Months Following an Epizootic.

Neutralization index ($\log_{10}$)	Serum HI titer with varying units of antigen 2	4	8
2.6	N*	N	N
3	320†	80	40
2	N	N	N
2.1	N	N	N
2	10	N	N
3	N	N	N
2.5	N	N	N
2.3	320	160	80
2.1	80	40	10
2.5	”	”	20
2.7	160	80	40
2.9	”	40	N
2.2	40	N	N
2.8	”		

* Negative at 1:10 serum dilution.

† Reciprocal of highest serum dilution causing complete inhibition.

Note: These sera were collected in areas of Maryland and Virginia where EEE was known to have occurred the previous year. Fifty-nine of the 73 sera tested had no measurable HI or neutralizing antibodies.

Discussion. Early detection of HI antibodies reported here is in agreement with findings of other investigators studying other arthropod-borne encephalitides(3,9,10). The HI test, therefore, is particularly well-suited for serodiagnosis of EEE since high mortality in this disease usually precludes the opportunity to obtain second serums with which an increase in antibody titer can be demonstrated. Unpublished results of more extensive use of the HI test in a recent EEE epizootic in the Middle Atlantic states further supports the latter observation. The problem of cross-reactions of EEE with other members of Group A Arbor viruses could conceivably be a problem in areas where other members of the group are prevalent. This would not be a serious problem in the U.S.A. where EEE and WEE are the only Group A members known to occur at present. Further, these 2 agents do not crossreact to a high degree(12, 13).

TABLE III. Results of Laboratory Tests Used to Diagnose EEE in 3 Horses.

Serology*		Examination of brain	
Neutralization index ($\log_{10}$)	HI titer	Virus isolation	Histopathology
1.8	1:640	Yes	Pos.†
1.3	”	No	”
1	1:2560	Yes	”

* Serum specimen obtained during acute stage of disease within 24 hr of death.

† Lesions characteristic of viral encephalitis.

The fact that none of the experimental horses developed clinical EEE may be attributed to several factors. Results of epidemiological surveys indicate that many horses undergo subclinical infections with EEE virus. Furthermore, other investigators(11) found that only 5 of 13 horses inoculated with EEE virus succumbed to clinical infection. The possibility also exists that the pathogenicity of this EEE virus strain for the horse was reduced by the number of mouse brain passages.

Summary. 1. Four horses inoculated with EEE virus remained asymptomatic following injection but did develop measurable HI and neutralizing antibodies as a result of infection. HI antibodies were detectable earlier than neutralizing antibodies but the levels tended to drop more rapidly. 2. Of 14 horses and ponies having significant levels of neutralizing antibody, 5 to 9 had measurable HI antibody depending on number of units of antigen employed in the test. 3. Sera from 3 clinical cases of EEE were positive on the HI test while only one of these animals had a significant neutralizing antibody level.

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Evaluation of Infection-Enhancing Activity of Modified Corticoids.[*][†]

(25593)

J. K. Frenkel

Dept. of Pathology and Oncology, University of Kansas School of Medicine, Kansas City, Kan.

Although the effects of corticoids concerning inflammation, glycogen deposition and sodium retention are commonly investigated, the important side-effects on infection(1) have not been studied systematically. It will be shown here, that by corticoid treatment in hamsters, acquired resistance to *Besnoitia,* an obligate intracellular protozoan(2), is easily abolished, resulting in fatal relapse of chronic infection. The speed with which relapse occurs is proportional to dose of corticoid employed. Infection-enhancing activity of a number of modified corticoids has been evaluated by means of this system.

Materials and methods. A. *Infection. Besnoitia jellisoni* was originally isolated from white-footed mice, *Peromyscus maniculatus artemisiae,* found naturally infected at upper Salmon River, Idaho(2). Cyst-forming capacity of the organism was eliminated by rapid passage (twice weekly) in peritoneal cavity of mice. The strain is now beyond its 550th passage. Six to 8 week old Golden hamsters, *Mesocricetus auratus,* of random-bred stock, are infected subcutaneously with peritoneal exudate containing a few thousand *Besnoitia.* To permit development of resistance with persistence of infection (premunition), hamsters are treated for 4 weeks with sodium sulfadiazine in drinking water, at 90 mg% for females and 180 mg% for males. (Fed on Purina Lab Chow only, at temperatures 70-80°F, adult female hamsters drink approximately 10 ml daily, but males drink only 5-6 ml.) They are tested for resistance by reinfection, subcutaneously, and virtually all animals survive, although they may develop endophthalmitis(3) and signs of chronic encephalitis(2). After another 3 or 4 weeks they are ready for assay of corticoids; only those few animals showing progressive encephalitis with weight loss are eliminated from the test. For comparative experiment *Toxoplasma gondii* (RH strain) is used in similar manner to produce chronic infection in hamsters. As a rule sulfadiazine treatment has to be given intermittently for 2-3 months till chronic latency has been achieved(4). B. *Steroids.* The following corticoids were used (numbers and abbreviations refer to Fig. 2): 1. cortisone acetate (Eac); 2. hydrocortisone (F); 3. prednisolone (Δ^1F); 4. prednisolone acetate (Δ^1 Fac); 5. 2-methyl-9-α-fluoro hydrocortisone acetate (2Me 9F Fac); 6. 6-methyl prednisolone (6 MeΔ^1 F); 7. 16-α-hydroxy-9-α fluoro-prednisolone (triamcinolone, 16 OH 9FΔ^1 F); 8. 9-α fluoro-prednisolone (9 FΔ^1 F); 9. triamcinolone acetonide (acetaldehyde derivative, 16 OH 9 FΔ^1 F actnd); 10. 16-α methyl-9-α fluoro-prednisolone (dexamethasone, 16 Me 9FΔ^1 F). Additional steroids are listed in Table I. C. *Tests.* After grinding in ground glass homogenizer, compounds were administered suspended in aqueous vehicle containing .5% sodium carboxy-methylcellulose, .4% polysorbate 80 (Tween), .9% sodium chloride and .9-1.5% benzyl alcohol. However, Δ^1 Fac was used as supplied in aqueous vehicle containing .5% phenylethyl alcohol and .01% benzalkonium chloride. Graded doses at 2-4 levels were injected subcutaneously, once daily, 6 times weekly. Generally 4-6 hamsters were used/dose level; identical groups in repeated tests were combined for computation of means. Fatal relapse results from confluent pneumonia with focal necrosis or from acute encephalitis. Commonly, there is proliferation of *Besnoitia* at sites of corticoid injection(5); the number of deposits provides an estimate of drug insolubility. Specificity of lesions is checked by impression smears, which

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[†] Grateful appreciation is expressed to the following for supplying steroids: Drs. C. H. Demos of Lederle Lab., W. E. Dulin and J. A. Hogg of Upjohn Co., J. Fried of Squibb Institute, E. Henderson of Schering Corp., H. J. Robinson and R. H. Silber of Merck Inst.

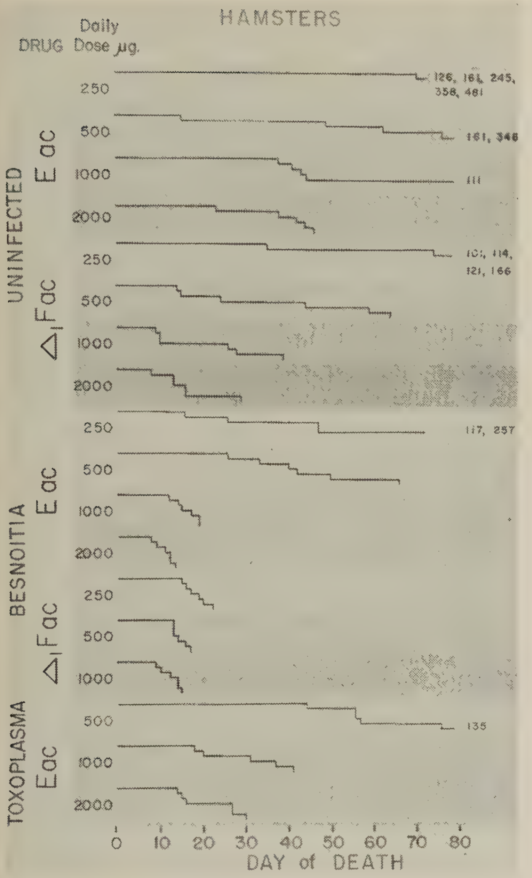

FIG. 1. Effects of cortisone (Eac) and prednisolone (Δ^1 Fac) on uninfected hamsters and on hamsters with chronic *Besnoitia* and *Toxoplasma* infection. Ordinates represent No. of animals (out of 6) surviving at each dose level. Note rapid and uniform mortality in hamsters with *Besnoitia* infection and treated with large doses of either corticoid. Also note that lower doses of corticoids will result in death at irregular and widely dispersed intervals (day of death beyond 80 given in numerals).

show numerous crescentic *Benoitia* organisms, visible at magnifications of 200-500, following Giemsa staining, clearing and mounting with a coverslip.

Results. A. *Corticoid sensitivity of normal hamsters, and of hamsters with chronic* Besnoitia *or* Toxoplasma *infections* (*Fig. 1*). It is apparent that infected hamsters are more sensitive to corticoids than uninfected ones. Administration of 2000 µg of Eac daily resulted in death after an average of 11 days for *Besnoitia*-infected, 22 days for *Toxoplasma*-infected and 35 days for uninfected hamsters. Resistance to *Besnoitia* was consistently more sensitive to cortisone than resistance to *Toxoplasma*. In similar doses, Δ^1 Fac was more effective than Eac in bringing about death of hamsters, whether infected with *Besnoitia* or not. Specific organisms could generally be found in the lung or brain lesion of infected hamsters. However, when death was delayed beyond one month, other lesions often occurred, such as caused death of "uninfected" animals. These lesions included: phagedenic ulcers (30%) originating from bites, sites of corticoid injection, or from large brown fat pads; enteritis with peritonitis (20%); meningitis (9%); liver or spleen abscesses (9%); and dissecting aortic aneurysms that had ruptured (11%). Bacteria that were cultured included coliforms, *Proteus, Mima, Staphylococcus* and diphtheroids. With higher corticoid dosages relapses of *Besnoitia* infection occurred within a few days in second or third week. It is this period that appeared to be most useful for assay purposes.

B. *Dose-response curves for natural and modified corticoids and derivation of potency ratios* (*Fig. 2*). The mean days of death at

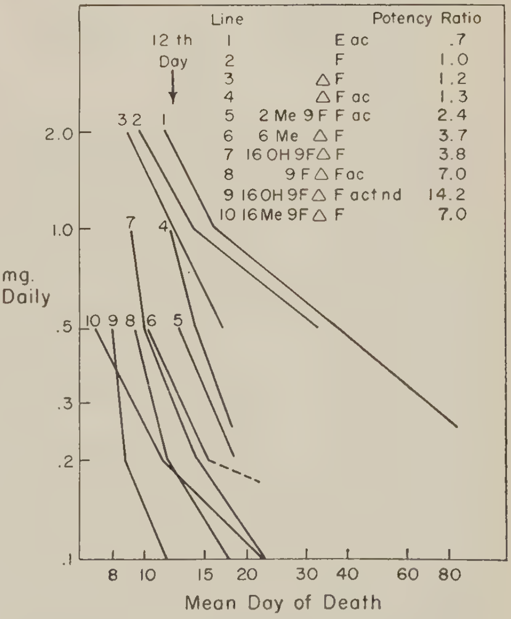

FIG. 2. Relapse of chronic *Besnoitia* infection in hamsters. Dose-response lines for 10 corticoids are identified by numbers. Both dose and time are charted logarithmically. Potency ratios for 12th day are given in inset.

TABLE I. Potency of Corticoids Relative to Enhancement of *Besnoitia* Infection in Hamsters. Tissue solubility was estimated from the number of steroid deposits remaining.

Steroids	Enhancement of infection	Solubility in tissue comp. to Eac
DOC (21-OH progesterone)	0 *	+ ¶
2-Me-11-OH "	0 †	— **
9-F-11-OH "	0 ‡	—
6-Me-11-keto- "	.6	±
6-Me-17-acetoxy- " (Provera)	0 ‡	—
9, 21-F-21-desoxy F	~1 §	—
9, 21-F-21-desoxy Δ^1 F	~2 §	+
Eac	.7	±
Δ^1 Eac	.8	±
F	1	+
Δ^1 F	1.2	+
Δ^1 Fac	1.3	—
9-F Fac	3.1	±
9-F Δ^1 Fac	7.0	++
2-Me F	~ .4‖	—
2-Me 9-F Fac	2.4	—
6-Me Fac	1.5	+
6-Me Δ^1 F	3.7	+++
16-OH-9-F F	1.6	±
16-OH-9-F Δ^1 F	3.8	+
" actnd	14.0	+++
16-Me 9-F Δ^1 F	7.0	+++

* Enhancement of infection tested up to 1 mg/day.

† Tested up to 1.5 mg/day.

‡ Tested up to 2 mg/day.

§ Steep curves, not intersecting 12th day.

‖ Active at 1 mg, not at 200 μg.

¶ +, greater solubility than Eac.

** —, lesser solubility than Eac.

2-4 dose levels were plotted logarithmically. Dose of drug that produced fatal relapse on 12th day (mean) was chosen for comparison of potency ratios. For hydrocortisone alcohol this is 1.3 mg (line 2) which serves as standard unit. Relative potency = 1.3/(mg of assayed corticoid required to produce death on 12th day). Ratios of enhancement of infection by all steroids tested are compared, and indications of drug solubility in tissue are given (Table I).

Discussion. A. *Model.* Hamsters are the most corticoid-sensitive small laboratory animal available (unpublished observations), being more susceptible to corticoid "toxicity" than mice, rats, and guinea pigs(6). Fig. 1 relates corticoid sensitivity of infected and control hamsters, with 73% of deaths even in the latter group accounted for by infection with indigenous flora. Similar to man, hamsters secrete hydrocortisone, unlike rats and mice that secrete corticosterone predominantly.

Chronic, latent *Besnoitia* infection is comparable to chronic tuberculous and fungal infections of man, which may either relapse, or be accentuated in their course by corticoid therapy if doses beyond the physiologic range are used. Acute and subacute *Besnoitia* infection is even more sensitive to corticoids than chronic infection utilized here(5). This infection system, which has been used for assay of compounds less potent than cortisone (7), may be representative of those acute viral infections, such as chickenpox and poliomyelitis, which are affected adversely by large doses of corticoids(1).

The action of corticoids on certain acute bacterial infections has been studied qualitatively(8,9,10) and quantitatively(11). However, excessive inocula resulting in rapidly fatal course were employed. Endotoxin effect, where present, may actually be moderated by corticoids. There is uncertainty as to which of a variety of intra- and extracellular factors affecting phagocytosis, digestion, or antibody mechanisms, are modified by corticoids, or whether their effect is on non-specific resistance or acquired immunity, which is unaffected in salmonellosis(13). In chronic *Besnoitia* infection organisms persist intracellularly, antibody is present (unpublished observations), and hamsters were shown to be immune to challenge. When pharmacologic doses of corticoids are given, organisms again start to multiply within cells that could previously curb this multiplication. Relapse can prove fatal to the host in approximately the same time as fatality results from primary acute infection not treated with sulfadiazine. A further indication of the corticoid-sensitivity of the hamster's resistance to *Besnoitia* is that chronic infection frequently leads to progressive and ultimately destructive adrenal involvement, apparently dependent on the endogenous hydrocortisone produced(5). Corticoid treatment did not affect stable antibody levels(14). Since hydrocortisone does not enhance proliferation of *Besnoitia* in non-immune hamsters, nor in hamster cell cultures (unpublished), and exerts its effect only after the presence of immunity had been indicated by resistance to challenge, a direct growth stimulation in the complete host, or an effect on natural resistance alone, appears unlikely.

B. *Comparison of infection-enhancing effects.* By definition the Potency Ratios are based on amount of corticoid required to produce death on 12th day. They have only a relative meaning, but are fairly consistent in terms of rank relationship. Type of curves obtained depends not only on structure of corticoid, but also on biological half-life, especially solubility and rate of degradation. 16-hydroxylated and methylated compounds have been reported to be more active by mouth than by subcutaneous administration(15). The latter route was used throughout this study.

A 50% mortality end point (LD_{50}) in the ordinary sense is difficult to derive, since mortality occurs as a continuum, and there is no meaningful break-off point (Fig. 1). If one plots mean days of death for a wide dose range of corticoids, one obtains a sigmoid curve (hyperbolic tangens). Asymptotes are present near the maximal obtainable drug effect (Fig. 2), limited by speed of biological responsiveness, as well as near minimal observable effects, approaching natural longevity. Near central part of curves parallelism could be observed. Usually, assays are conducted in this range. In our model, however, the variation in response is too great in this segment of curve (*cf.* Fig. 1, 250 and 500 μg Eac) and testing would need to be prolonged. In the interest of speed, with but slight variation in response, an end point in the second week was chosen, although curves are not parallel in this range. This corresponds to a 50% mortality end point for the 12th day, $LD_{50(12)}$, "lethal dose" referring to the corticoid.

C. *Are certain corticoids less apt to enhance infection from a clinical point of view?* Since glucocorticoid and anti-inflammatory activities, when both assayed in the same host showed divergent ratios in the highly potentiated compounds(16,17), there can be little doubt that different mechanisms of action are involved. Chemical modifications which manipulate this divergence and which should aid in defining biologic mechanisms of action, have been found in the modified progesterone and hydrocortisone series(18,19). The possibility that mechanisms of infection-enhancement and of anti-inflammatory activity might also differ, suggested that certain compounds might have improved therapeutic indices in man when anti-inflammatory effect is desired. Analysis of this problem necessitates comparison of both activities in either hamster or man.

Summary. (1) Chronic latent *Besnoitia* infection in Golden hamsters is highly sensitive to corticoid effects. (2) Fatal relapse from renewed microbial proliferation can be brought about as early as 6 days after initiation of corticoid therapy. (3) By assay which uses as end point the dose producing relapse on 12th day, potency ratios have been determined for 22 modified progestins and corticoids.

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Growth Inhibition and Leg Disorder in Chicks Following Parenteral Administration of Hemin and Non-Metalloporphyrins.* (25594)

UNABELLE B. BLACKWOOD† AND WILLIAM O. POLLARD (Introduced by M. S. Shorb)
Dept. of Poultry Husbandry, University of Maryland, College Park

The major portion of heme in the animal body is found as the iron-porphyrin portion of hemoglobin. It is also found in molecules of some very important enzymes such as catalases, peroxidases, and cytochromes. In recent years considerable information has been presented concerning synthesis of heme and its porphyrin precursors(1,2). It is known that many co-factors, such as pyridoxal phosphate (Vit. B_6), coenzyme A (thus pantothenic acid) and others are required to synthesize heme and porphyrins from succinic acid and glycine. It is also known that 2-ethyl-5-methylbenzimidazole (EMB) inhibits the synthesis of heme in chicken erythrocytes (3,4). However, very little is known about the metabolic steps or co-factors involved in the catabolism of heme or related porphyrins. This paper demonstrates that in chick, hemin (heme chloride) is unable to reverse inhibition of EMB and that hemin itself and non-metalloporphyrins have inhibitory effects resulting in growth depression and leg deformity.

Methods and materials. Day-old chicks of the strains and crosses indicated in Table I were weighed and distributed into groups of 18 or 20 of equal average weights in wire batteries (avg. starting wt. all exp. = 42.4 g) and placed on a practical starter ration. They were fed *ad libitum* and a constant light source was supplied. Materials used were 2-ethyl-5-methylbenzimidazole (supplied by Dr. Karl Folkers, Merck & Co.); hemin (recrystallized) Eastman Kodak Co.; hematoporphyrin dihydrochloride (recrystallized) and protoporphyrin (C.P.), Mann Research Laboratories, Inc. In 2 experiments hemin was dissolved in 0.1 N NaOH and EMB was dissolved in 0.07 N HCl. In the remaining experiments, hemin and the porphyrins were dissolved in 1% NH_4OH and pH of the solution adjusted to 7.5 to 8.0 with glacial acetic acid. The volume was then adjusted with distilled water so that each 0.2 cc contained either 3 or 5 mg. Controls consisted of an equal number of birds as experimental groups; one group received no treatment, and the other received solvent injections. A total of 5 daily injections of 0.2 cc were given in the area of loose connective tissue between the medial surface of the femur and body wall beginning at 2 days of age. Hematocrits were determined on individual chicks at termination of experiments using standard Wintrobe hematocrit tubes. Chicks were weighed individually at 5, 10, and 15 days after beginning of treatment in all experiments and also at 21 days in 3 of these experiments (Table I).

Results. That hemin has inhibitory activity in the chick was found when it was administered with EMB to New Hampshire-Columbian females to test its reversing ability. Table I shows that hemin not only did not reverse the small but significant weight depression of EMB, but severely depressed growth below that of EMB alone. A large proportion of chicks which survived EMB-hemin treatment developed outwardly bowed legs similar to that found in certain nutritional deficiencies such as choline, Mn, biotin and others. However, birds did not develop "slipped-tendon" as seen in classical perosis. Injection of 3 mg hemin with and without 1.5 mg EMB into White Leghorn chicks of both sexes showed that hemin alone was as effective a growth depressant as the combination of hemin-EMB (1-8 day weight gains‡ : 45.2 g solvent control; 31.7 g hemin-EMB; 31.1 g hemin). Additional experiments (Table I) confirmed the indication that subcutaneously administered hemin adversely affects chick

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† Present address: Poultry Science Dept., Ohio State Univ., Columbus.

‡ Results of preliminary experiment, data not included in table. Four injections given over 8 day period.

TABLE I. Average 5 Day Period Weight Gains and Leg Disorder Observed in Various Strains of Chicks Following Treatment with EMB, Hemin and Certain Non-metalloporphyrins. Mean ± S.E. of difference from control is indicated where calculated. Doses indicated are daily doses, administered for 5 days.

Treatment	Days of exp. 1-5	6-10	11-15	16-21	Bowed legs (% survivors)	Mortality, %
	g gain/period					
New Hampshire-Columbian Females (20 chicks/treatment)						
None	15.9	33.0	60.2	142.8	0	0
Solvent (HCl)	15.8	35.0	58.2	148.2	0	0
EMB, 1.5 mg	11.0 ± 1.21*	30.2 ± 2.01	59.0 ± 2.59	146.1 ± 3.84	0	5
Idem + hemin, 5 mg	4.6 ± 1.31*	22.3 ± 3.01*	51.7 ± 4.09†	161.2 ± 5.13	36.0	45
Vantress-Columbian Males (18 chicks/treatment)						
None	25.8	44.4	84.4	78.9	0	0
Solvent (NH_4OH)	27.1	42.5	82.6	80.5	0	0
Hemin, 3 mg	17.8 ± 1.2*	31.4 ± 1.9*	75.0 ± 3.2*	77.8 ± 2.6	0	0
" 5 "	13.6 ± 1.8*	31.0 ± 1.7*	74.4 ± 3.6*	74.4 ± 3.1	0	0
Vantress-Columbian Females (18 chicks/treatment)						
None	28.0	43.2	78.2	82.3	0	0
Solvent (NH_4OH)	25.9 ± 1.9	45.8	77.6	75.5	0	0
Hemin, 3 mg	18.8 ± 1.8*	33.9 ± 2.1*	73.8 ± 3.1	74.1 ± 3.4	11.1	0
" 5 "	15.8 ± 1.9*	31.4 ± 2.1*	75.9 ± 3.2	69.3 ± 3.1	35.3	0
Hematoporphyrin, 2 mg‡	24.4 ± 2.1	31.9 ± 2.7*	66.1 ± 3.8*	69.5 ± 7.2	0	0
White Rock Males (20 chicks/treatment)						
None	27.2	36.2	85.0		0	10
Hemin, 5 mg	10.3 ± 1.9*	46.3 ± 3.4*	71.7 ± 4.6*		35.3	15
White Rock Males (20 chicks/treatment)						
None	37.8	45.7	67.3		0	0
Solvent (NH_4OH)	34.3	48.1	71.0		0	5
Hemin, 5 mg	23.1 ± 2.4*	29.5 ± 4.8*	56.3 ± 6.8†		33.0	40
Hematoporphyrin, 5 mg	16.5 ± 2.9*	20.0 ± 5.4*	43.4 ± 6.2*		0	45
Protoporphyrin, 5 "	22.8 ± 2.0*	30.7 ± 3.5*	48.5 ± 5.2*		29.4	15

* 0.1%-2.0% level of significance (*t* test). † 2.5%-5.0% level of significance (*t* test).
‡ Group contained 14 chicks. Treatment started day 3.

growth and leg development.

Very little difference between 3 and 5 mg of hemin was noted in growth depressant effect, although 5 mg was considerably more effective than 3 mg in producing bowed legs. Recovery from hemin treatment, as indicated by weight gains at progressive periods, was not complete at 15 days (10 days after cessation of administration) in any experiment except one involving Vantress-Columbian females.

Experiment 5 showed that protoporphyrin has effects similar to hemin with respect to growth depression and bowing of legs, and that hematoporphyrin (Exp. 3 & 5) depresses growth to the same or greater extent than hemin and protoporphyrin, but fails to cause bowed legs. Hematoporphyrin acted over a longer period of time than hemin in each of 2 experiments in which it was tested.[§] This was indicated by lower weight gains during 11-15 day period.

Bowed legs were found only in females of the Columbian crosses. However, this condition was not restricted to females since White Rock males (Fig. 1) developed bowed legs to as great an extent as Columbian cross females. Fig. 1 also shows that the left leg appears more severely involved, which was noted throughout all experiments but was most prominent in experiments with Columbian cross females in that bowing of legs was confined solely to the left leg.

Solvent control injections did not depress

[§] Since manuscript was written, additional experiments (Pollard, Shorb, unpublished) indicate that hemin incites a foreign body reaction at injection site, consisting of a walling off of injected substance. This process would make toxicity of hemin of shorter duration than porphyrins which do not incite this reaction, and also account for longer period of activity of hematoporphyrin and protoporphyrin.

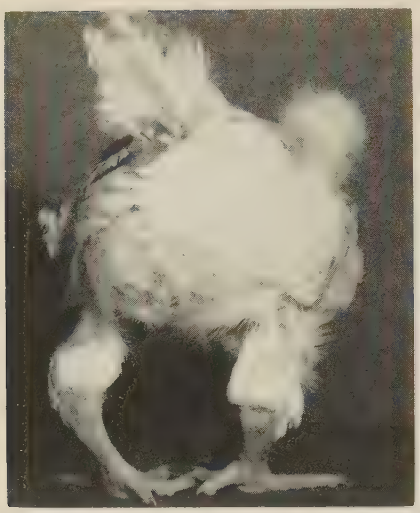

FIG. 1. White Rock male 10 days following 25 mg hemin treatment. Note outward bowing of legs, more severe in left.

chick growth substantially below that of non-treated groups (Table I). Thus dilute HCl, NaOH and NH_4OH cannot account for the adverse effects found in the chicks receiving EMB, hemin or the porphyrins. Gains during 1-10 days with the latter compounds were lower than controls regardless of treatment.

Determinations of hematocrits at both 16 and 21 days revealed no significant effect of hemin or hematoporphyrin on the packed cell volume (29.5-32.4%).

Discussion. This study confirms conclusively our previous report(5) that parenteral administration of hemin and hematoporphyrin has adverse effects on chick growth and hemin causes an outward bowing of legs. It shows, in addition, that protoporphyrin also affects chick growth and produces a similar bowing of legs.

The mechanism of hemin, proto- and hemato-porphyrin inhibition in the chick is unknown. It is evident, however, that the effects of hemin cannot be attributed to the presence of iron since its metal-free porphyrin homologue, protoporphyrin, has similar effects. Hematoporphyrin, differing from protoporphyrin by having 2 hydroxyethyl side chains in place of 2 vinyl chains, failed to cause bowed legs. The inhibition does not appear to involve formed blood elements quantitatively as indicated by hematocrit, since packed cell volume was not affected. Although Vannotti *et al.*(6) found that protoporphyrin administered intravenously into the normal rabbit had no effect on hemoglobin synthesis, they found it caused a slight increase in liver cytochrome *c*. It is possible that the higher levels of hemin and protoporphyrin used in this study cause a metabolic imbalance in the chick by increasing cytochrome *c* and that the chick is less able to metabolize exogenous hemin and porphyrins than mammals.

Sex difference in leg bowing could not be attributed to difference in growth rate or to differences in recovery from hemin and, therefore at present, is unexplained. Since the bowed leg condition resembles that resulting from several nutrient deficiencies, it is possible that catabolic breakdown of this additional hemin and protoporphyrin requires certain metabolites to a higher degree than were supplied in the diet, thus resulting in nutrient deficiencies. Or, on the other hand, it may be that their presence blocks some metabolic step or prevents utilization of available metabolites. If these hypotheses are true, inhibitory effects of hemin and porphyrins in the chick could serve as a means for studying metabolic steps and co-factors required for their catabolisms, or for studying other metabolic processes in the chick.

Summary. In this study on 2-day old chicks the reversing effects of hemin on 2-ethyl-5-methylbenzimidazole and growth depression and leg deformity produced by hemin and certain non-metalloporphyrins were investigated. Hemin did not reverse the growth depressing action of EMB. Hemin and porphyrins, proto- and hemato-porphyrin, caused depression of growth below that of controls; also, hemin and protoporphyrin caused bowing of legs. That the basic porphyrin nucleus is the probable cause of this depression and leg bowing and not iron imbalance is demonstrated by similarity of action of protoporphyrin and hemin. Hematoporphyrin caused growth depression but did not produce leg bowing. Incidence of bowing was directly related to dose. Hematocrits determined at 16 and 21 days were not affected by treatment.

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Liver Ketogenesis. II. Role of Glucose Cycloacetoacetate on Ketogenesis in Hyper- and Hypothyroid Rats.* (25595)

M. C. NATH AND J. M. KHANADE
Dept. of Biochemistry, Nagpur University, Nagpur, India

The authors reported that hydrolyzed glucose cycloacetoacetate (GCA) when added to liver slices in presence of pyruvate and acetate enormously decreases formation of acetoacetate(1) and increases glycogen(2). Because thyroid feeding (reviewed by Barker(3)) causes enormous rise in production of acetoacetate in the liver, we attempted to find out the role of hydrolyzed GCA in preventing excess of ketogenesis in liver induced by thyroid feeding. We also studied the effect of thiouracil and the combined effect of thiouracil and GCA on acetoacetate formation from lower fatty acids in presence of liver slices. Utilization of acetoacetate by kidney slices in different groups of animals has also been measured.

Method. In view of relatively rapid response of young rats to thyroid and thiouracil feeding(4), male albino rats weighing 75 g to 100 g and growing on standard diet, were used. The animals were divided into 6 groups of 6 rats each and fed the following basal diet *ad lib.* for 6 weeks:

	%		%
Wheat flour	10	Salt mixture	5
Sucrose	49	Yeast powder	4
Casein	20	Marmite	2
Groundnut oil	10	Cod liver oil	.5

Rats in Group I received basal diet and Group II basal diet plus freshly hydrolyzed GCA, 0.8% of diet by weight. Desiccated thyroid (0.3% of diet by wt) was mixed in diet of animals in Groups III and V and thiouracil 0.3% in Groups IV and VI as these concentrations were effective(Hander,5). Rats in Groups V and VI received 0.8% GCA hydrolyzed daily in addition to thyroid and thiouracil respectively.

Tissue preparation and analytical methods. At the end of 6 weeks, animals were stunned by a blow on head and decapitated. Liver and kidneys were taken out immediately and placed in ice-cold saline. Tissue preparations were done as described by Umbreit *et al.*(6). Liver and kidney slices were cut with razor blade, suspended in conventional Warburg vessels containing 3 cc of Krebs' Ringer Phosphate buffer of pH 7.2. The vessels were gassed for 1 minute with oxygen, then incubated for 2 hrs. at 37.5°C with constant shaking. Then acetoacetate was estimated from media in vessel according to manometric method of Edson(7). Liver glycogen estimations were done by the method of Good *et al.*(8) and blood sugar by that of Hagedorn and Jensen(9). GCA, a crystalline product obtained by condensation of glucose with acetoacetate, was prepared according to the method of West(10) modified by Nath *et al.*(11). It was hydrolyzed with 2N HCl for 12 min and extracted with ether to remove resinous materials formed. Aqueous solution was adjusted to pH 7.2 by adding requisite amount of 2N NaOH, then fed to the animals. Total fat content of liver was extracted thrice with alcohol-ether mixture (1:1). The extract was

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* The authors record their thanks to the Council of Scientific and Industrial Research for a Research Grant.

TABLE I. Body Weight Gain, g/100 g/Week, of Different Groups of Rats.

Group	No. of animals	Period in wk						Avg food intake, g/rat/day
		1	2	3	4	5	6	
Normal	6	8	16.1	24.4	32.2	37.7	45.2	10
Hydrolyzed GCA fed	6	7	13.2	25.2	37.7	47.7	55.2	10
Thyroid fed	5	6	12	17.6	19.6	21.6	21.6	15
Thiouracil fed	6	7	14	20	25	27.5	30	8
Thyroid + hydrolyzed GCA	6	5	10	14.6	19.1	20.9	21	12.5
Thiouracil + hydrolyzed GCA	6	5	10	14.6	18.6	22.9	24.9	8

evaporated to dryness and the remaining solid reextracted with petroleum ether (40-60° B.P.). Petroleum ether was evaporated to dryness and amount of dry fat calculated. At end of every week, rats were weighed and average food consumption in g/day/rat was calculated. Urine was tested qualitatively for sugar and ketone bodies.

Results. Blood sugar values (Table II) indicate that hydrolyzed GCA feeding to hyper- and hypothyroid rats, Groups V and VI ($t = 2.3$ and 1.7 respectively) has a hypoglycemic effect when compared to respective control Groups III and IV. In normal rats, however, it does not seem to affect blood sugar level significantly ($t = 1.05$).

Carcass lipids(12) and liver glycogen are very low in hyperthyroid animals(13, 14) but feeding of hydrolyzed GCA to Group V causes a significant increase in liver glycogen as compared to Groups III, IV & VI. Beneficial effect of GCA on glycogenesis in liver and diaphragm has already been reported(2). Feeding of hydrolyzed GCA to normal animals (Group II) increases total fats in liver to some extent as compared with normal (Group I).

Gross enlargement of liver and kidney in hyperthyroid animals agrees with observations of other workers(15,16) and hydrolyzed GCA feeding to these rats (Group V) prevents such enlargement. Hypertrophy of kidney in hypothyroid rats is also prevented by feeding hydrolyzed GCA (Group VI).

Table III indicates oxygen consumption and acetoacetate production by the liver slices from different groups. That general metabolism is accelerated considerably in hyperthyroid animals has already been shown(3). Increased oxygen consumption and acetoacetate production in Group III confirm these findings. In this group, liver produces large amounts of acetoacetate though there is some increase in utilization by kidney slices (Table IV). Acetoacetate produced in Group II is significantly less than that in normal rats (Group I) and hydrolyzed GCA feeding prevents excess of ketogenesis by liver slices as seen by comparing results of Groups III and V. Endogenous acetoacetate production in kidney is high in hypothyroid rats as observed by Mookerjea and Sadhu(17). Lower rate of oxygen consumption and acetoacetate production in liver of hypothyroid rats indicates slowing of metabolism. Utilization of acetoacetate by kidney slices of animals fed thy-

TABLE II. Blood Sugar, Liver Glycogen, Liver Fat, % of Tissue Weights (Wet Wt) of Different Groups of Rats.

Group	No. of animals	Blood sugar, mg/100 cc	Liver glycogen, g/100 g	Liver fat, g/100 g	Wet wt of tissue, g/100 g body wt	
					Liver	Kidneys
Normal	6	86 ± 2	2.18 ± .2	3.5 ± .3	3.6 ± .18	.82 ± .04
Hydrolyzed GCA fed	6	78 ± 3.5	2.14 ± .14	4.04 ± .28	3.61 ± .13	.70 ± .03
Thyroid fed	5	100 ± 3	.21 ± .10	4.3 ± .31	5.2 ± .2	1.10 ± .05
Thiouracil fed	6	90 ± 2	3.0 ± .25	4.0 ± .28	4.01 ± .1	.92 ± .04
Thyroid + hydrolyzed GCA	6	83 ± 4	1.65 ± .21	4.4 ± .21	3.43 ± .11	.96 ± .03
Thiouracil + hydrolyzed GCA	6	80 ± 3	3.6 ± .2	4.4 ± .23	4.0 ± .12	.82 ± .04

TABLE III. Oxygen Uptake and Acetoacetate Production by Liver Slices from Different Groups of Animals.

Group	No. of animals	Oxygen uptake in μl/100 mg wet wt of liver slices in 2 hr			Acetoacetate produced in *μl/100 mg of liver slices, wet wt, in 2 hr		
		No substrate	Butyrate, .017M	Acetate, .08M	No substrate	Butyrate, .017M	Acetate, .08M
Normal	6	230 ± 12	354 ± 15	276 ± 20	15 ± 6	85 ± 10	60 ± 13
Hydrolyzed GCA fed	6	240 ± 15	365 ± 17	265 ± 19	12 ± 7	81 ± 11	38 ± 13
Thyroid fed	5	280 ± 12	400 ± 20	338 ± 18	65 ±15	240 ± 16	280 ± 25
Thiouracil fed	6	153 ± 10	251 ± 15	190 ± 12	30 ±11	46 ± 12	33 ± 6
Thyroid + hydrolyzed GCA	6	209 ± 15	386 ± 20	283 ± 21	29 ± 7	102 ± 20	67 ± 11
Thiouracil + hydrolyzed GCA	6	158 ± 11	190 ± 10	162 ± 9	6 ± 4	46 ± 5	15 ± 6

Krebs' Ringer phosphate buffer pH 7.2; incubation temp. 37.5°C; oxygen as gas phase.

* CO_2 in μl produced after decarboxylation of acetoacetate with aniline citrate.

roid and thiouracil respectively along with hydrolyzed GCA (Groups V and VI) is considerably increased.

Traces of sugar and ketone bodies were found in urine of animals after feeding thyroid for 3 weeks (Group III). Urine of other groups was free from sugar and ketone bodies.

Discussion. Results indicate acceleration of metabolic process in hyperthyroid and reduction in hypothyroid rats as previously described(3). Polyphagia and inhibition of gain in weight in hyperthyroidism, is known(18) so also the effect of thiouracil *i.e.*, lower metabolic rate and retardation of growth as described by Astwood *et al.*(19,20). Higher rates of acetoacetate production, its utilization and oxygen consumption found in hyperthyroid rats can be attributed to increase in metabolism, while the reverse is true of hypothyroidism. However, increased rate of endogenous acetoacetate production by kidney slices of hypothyroid animals, also observed by Mookerjea *et al.*(17), cannot be explained satisfactorily.

The significant effect of hydrolyzed GCA in preventing ketogenesis and accelerating acetoacetate utilization by kidney slices in different groups is evident from our results.

Ketogenesis is intimately related to lipogenesis and glycolysis. Thus Lyon *et al.*(21) showed that ketogenesis is inversely proportional to long chain fatty acid synthesis.

Liver slices in presence of hydrolyzed GCA synthesized fat from acetate at a higher rate than in control *i.e.*, without hydrolyzed GCA (unpublished observation). Thus, there is every possibility of hydrolyzed GCA acting to divert the acetyl-CoA, towards lipid synthesis, thereby reducing amount available for its condensation to form acetoacetate.

Summary. Feeding of hydrolyzed glucose cycloacetoacetate (GCA) promotes growth in normal animals and prevents excess of acetoacetate production by liver slices in normal as well as hyperthyroid rats. It accelerates acetoacetate utilization by kidney slices in

TABLE IV. Acetoacetate Used by Kidney Slices. Krebs' Ringer phosphate buffer pH 7.2; incubation temp. 37.5°C; oxygen as gas phase.

Group	No. of animals	Oxygen uptake, μl/100 mg kidney slices wet wt of tissues in 2 hr		Acetoacetate, μl/100 mg/2 hr by kidney slices	
		No substrate	Acetoacetate, 300 μl	Produced (without substrate)	Used 300 μl acetoacetate
Normal	6	560 ± 20	687 ± 31	Negligible	84 ± 14
Hydrolyzed GCA fed	6	514 ± 16	762 ± 17	”	185 ± 19
Thyroid fed	5	658 ± 16	917 ± 30	15 ± 4	118 ± 15
Thiouracil fed	6	459 ± 17	530 ± 18	33 ± 4	58 ± 15
Thyroid + hydrolyzed GCA fed	6	620 ± 20	731 ± 28	12 ± 3	147 ± 25
Thiouracil + hydrolyzed GCA fed	6	530 ± 12	630 ± 18	8 ± 3	90 ± 7

normal as well as in hyper- and hypothyroid rats. Higher percentage of liver glycogen and lower blood sugar was found in animals fed with hydrolyzed GCA, as compared to their appropriate controls.

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Urinary Excretion of Acid Mucopolysaccharides by Rabbits Injected with Chelating Agents and with Chondroitin Sulfate.*† (25596)

WILLIAM S. SMITH AND GRACE P. KERBY
Department of Medicine, Duke University Medical Center, Durham, N. C.

Of recent years there has been interest in treatment of scleroderma by chelation(1-4). The therapeutic efficacy of this approach is yet to be established. However the possibility of connective tissue being changed by chelators is intriguing. The present pilot study was undertaken in rabbits prior to studying humans to see if a change in excretion of acid mucopolysaccharides in urine could be detected following administration of chelating agents. Bryant, Leder and Stetten(5) have shown quantitation of urinary acid mucopolysaccharides to be a potentially rewarding approach to reflection of experimental alteration of connective tissue in rabbits, as demonstrated by their data on urinary levels following injection of papain intravenously. Their observations convey the implicit assumption that proteolytic enzyme as administered could affect connective tissue extravascularly and that chondroitin sulfate presumably released from protein binding *in situ* could then pass intravascularly to the kidneys where it could be excreted at least in part in the urine. In the present study it is shown that chondroitin sulfate† injected in large amounts intravenously into rabbits resulted in excretion of a small proportion but nonetheless a large amount of chondroitin sulfate in urine. When chelators‡ were administered extravascularly to rabbits, a transient and frequently mild rise in urinary excretion of acid mucopolysaccharides was noted; time of onset and degree and

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† Commercial chondroitin sulfate (General Biochemicals) of cartilaginous source, containing 15 to 20% protein.

‡ Disodium EDTA, with and without calcium furnished kindly as Endrate by Abbott Research Labs., North Chicago, Ill.

duration of rise was highly variable from animal to animal.

Method. Healthy male albino rabbits were used. Weights varied from 1.9 to 2.7 kg at time of injection, except the 2 animals receiving repeated injections of chondroitin sulfate, which weighed 4 kg. Baseline 24 hour urine collections were made under toluene on 7-11 days during 10-14 days preceding injection. I. Four rabbits received a single intravenous injection of 1000 mg of chondroitin sulfate in 5 ml saline. 24-hour urine collections continued thereafter. At 48 and 72 hours after injection, 2 rabbits received repeated intravenous injections of chondroitin sulfate, 1000 mg. II. Three rabbits[§] received a series of 3 subcutaneous injections of Disodium Endrate, Abbott, 500 mg in 5 ml[||] daily on successive days, with production of an intense and hemorrhagic local reaction sometimes with necrosis. III. Five rabbits[§] (Group III A) received 3-10 successive daily subcutaneous injections of Disodium Calcium Endrate, 500 mg in 5 ml,[||] without apparent local reaction. Two rabbits (Group III B) received similar injections daily for 10 days and simultaneously daily intramuscular injections of cortisone (Cortone Acetate, Sharp & Dohme), 12.5 mg in 0.5 ml for 10 days, for suppression of any inapparent inflammatory reaction. In all instances 24 hour urine collections were continued for about 15 days following completion of injections. Urine samples were held under toluene at 5°C until used for analysis. Acid mucopolysaccharides were recovered from duplicate 3-5 ml aliquots of urine by modification of the method of DiFerrante(6). After overnight dialysis against running tap water and precipitation with cetyltrimethylammonium bromide at pH 5.0, the centrifuged acid mucopolysaccharide complex was resuspended in a small portion of the supernate and dissociated from the quaternary amine with an equal volume of salt-saturated ethanol. The acid mucopolysaccharides were then reprecipitated with 4 volumes of ethanol and dissolved in demineralized water for measurement of hexuronic acid content by the carbazole method of Dische (7). Standard curves and criteria for acceptance were as defined previously(8). Repeated recoveries of 76-89% of 200 μg of chondroitin sulfate added to the original urine aliquot were demonstrated.

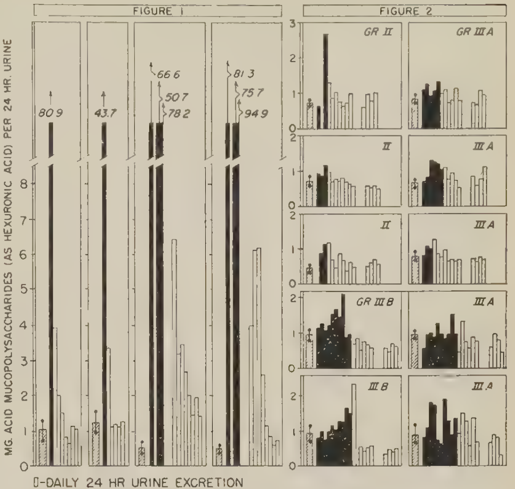

FIG. 1. 24-hr urinary excretions of acid mucopolysaccharides in rabbits (Group I) inj. intrav. with chondroitin sulfate.

FIG. 2. 24-hr urinary excretions of acid mucopolysaccharides in rabbits inj. subcut. (Group II) with Disodium Endrate (Abbott), (Group III A) with Disodium Calcium Endrate (Abbott), and (Group III B) with Disodium Calcium Endrate (Abbott) and Cortone Acetate (Sharp & Dohme).

Results. The acid mucopolysaccharides recovered were entirely similar in metachromatic and chromatographic characteristics to those described earlier(8) in human urine; extensive qualitative characterization has been deferred.

Fig. I records excretion of acid mucopolysaccharides in urine following single and repeated injections of 1000 mg of chondroitin sulfate intravenously. Total excretions of acid mucopolysaccharides during the 2-4 days required to return to baseline levels following a single injection of chondroitin sulfate indicated excretion of the order of magnitude of 5-10% of the injected amount. The sharp drop in excretion during 24-48 hour period following first injection in the 2 rabbits receiving 3 repeated injections indicated the probability of a similar pattern of excretion in these animals. On completion of the 3 injections in these rabbits, however, increased excretion was maintained in some degree during entire 12-day period of continued observation.

[§] Some animals had received Disodium Endrate and/or cortisone in a limited series of observations about 3 weeks prior to present studies. Baseline collections recorded here were made in 10 days immediately preceding present injections.

[||] Without further 200 X dilution recommended by Abbott Research Labs. for intravenous administration to humans.

Fig. II summarizes data in animals receiving Disodium Endrate and Disodium Calcium Endrate subcutaneously. A subsequent increase in excretion of acid mucopolysaccharides was noted at some time in all 10 rabbits thereafter, but onset and degree and duration of apparent response varied greatly from animal to animal. There was no significant difference in this response in the various groups of rabbits despite the fact that Group II (Disodium Endrate) exhibited intense local tissue injury, Group III A (Disodium Calcium Endrate) showed no apparent local reaction, and Group III B additionally received cortisone for suppression of any inapparent inflammatory reaction. All animals were handled equally on all injection and weighing days.

Daily volumes and specific gravity of urines were the only other parameters measured. These did not correlate in any way with levels of acid mucopolysaccharide excretion.

Discussion. The sharp rise in urinary excretion of acid mucopolysaccharides following intravenous injection of chondroitin sulfate into rabbits confirms the assumption of Bryant, Leder and Stetten(5) that a similar rise noted by them following intravenous injection of papain could have been caused by elevated level of chondroitin sulfate in the circulating blood of their rabbits. A comparison of quantitative data in the 2 studies further strengthens this assumption. In the present study, the general order of magnitude of excreted chondroitin sulfate in urine was 5-10% of total amount injected. In the previous study(5), serum levels rose by about 700-1100 μg/ml serum in rabbits weighing 800-1000 g and hence with circulating serum volume of perhaps 30-40 ml, the total rise in acid mucopolysaccharides therefore reflecting appearance in total circulating blood of an amount in the order of magnitude of 20-45 mg. Average increase in urinary excretion noted thereafter by the authors was 3.3 mg. Thus the percent excretion in their studies would appear to have been of the same order of magnitude as noted in the present studies where chondroitin sulfate was introduced directly into the blood stream. The fate of the remaining 90% was not determined. Following 3 successive injections of chondroitin sulfate, urinary excretion no longer returned promptly to the baseline in 3-5 days as after a single injection but was still somewhat elevated when urine collections were discontinued 12 days after the third injection. Levels were still 5-fold greater than baseline levels one week after the third injection.¶

The observation that about 5 to 10% of chondroitin sulfate introduced into the circulating blood of rabbits appeared in the urine does not warrant the assumption that elevated urine levels necessarily reflect an increased blood level of acid mucopolysaccharides. Indeed in humans nothing is yet reported regarding the fate of acid mucopolysaccharides introduced into the blood stream, other than as is apparent that urinary excretions rise sharply in patients receiving heparin. Jaques, Napke and Levy(10) present the protocol on one human subject given heparin intravenously. This individual showed total metachromatic activity in urine equivalent to 5% of amount injected.

Introduction of chelating agents subcutaneously in rabbits was followed by a transient and frequently mild increase in urinary excretion of acid mucopolysaccharides. Although levels of acid mucopolysaccharides in human plasma have been shown to rise nonspecifically in the presence of inflammation (11), the increased urinary excretion observed here in rabbits did not correlate grossly with degree of apparent local tissue injury which ranged from obvious intense injury with necrosis to no apparent inflammatory reaction and suppression of any inapparent inflammation by adrenocorticosteroids. Thus the possibility must be entertained that increased excretion was related to introduction of chelating agents. The observation warrants extension of studies to humans where use of chelators may offer an additional experimental approach to a study of factors influencing urinary levels of acid mucopolysaccharides in humans. The mechanism by which chelators may have induced an increased level of excretion in rabbits is not known. Whether the same observation of increased excretions holds in humans will be investigated.

¶ One of each pair of animals was sacrificed at 3 and 7 weeks, respectively, following either the last or only injection of chondroitin sulfate. Histologic examination of kidneys and other tissues, using the acridine orange fluorescent technic of Hicks and Matthaei(9), failed to reveal any unusual accumulations of fluorescent material. Cartilage from the same animals fluoresced brilliantly, as described for acid mucopolysaccharides in(9).

Summary. (1) Introduction of chondroitin sulfate into the blood stream of rabbits was followed by elevated excretion of acid mucopolysaccharides in urine totalling 5-10% of injected amount over 2-4 days thereafter. Repeated injections were followed by excretions which were still 5-fold greater than baseline levels one week after third and last injection. (2) Repeated subcutaneous injection of disodium EDTA and disodium calcium EDTA into rabbits was followed by a brief and frequently mild rise in urinary excretion of acid mucopolysaccharides; onset, degree and duration of rise varied widely from animal to animal.

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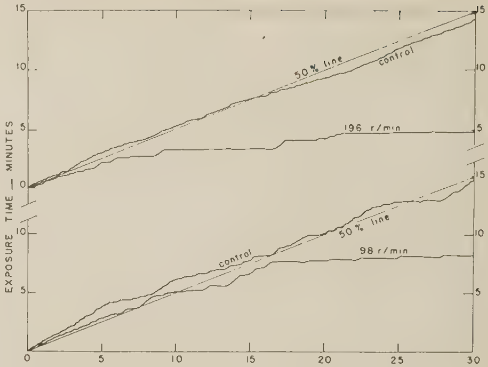
Radiation Avoidance in the Mouse. (25597)

HOWARD L. ANDREWS AND LOUIS M. CAMERON (Introduced by Willie W. Smith)
Radiation Branch, National Cancer Institute, N.I.H., P.H.S., Dept. of H.E.W., Bethesda, Md.

It is commonly stated that animals are incapable of detecting high energy radiation with the unaided senses but there is some evidence that it can be sensed under certain circumstances. Two individuals who received a substantial beta particle dose to the hands reported a local "tingling sensation" during exposure. Another individual receiving several thousand rads from an electron beam experienced a burning sensation although there was probably no temperature rise greater than 0.05°C. In animals relatively small doses of penetrating radiation have produced later avoidance behavior(1) in cats, rats, and mice. A direct radiation avoidance has been reported(2) in albino rats at dose rates of 1-4 r/minute after a total dose of 360 r had been delivered. Our experiments were designed to determine whether mice were capable of sensing the presence of an X-ray beam, and if so, whether it was recognized as something sufficiently unpleasant to be avoided.

Methods. During each run the test animal was in a plastic cage 25 x 9 x 6 cm pierced with many small holes to permit free air circulation. This cage was mounted on a knife edge so that it could tilt freely through a small angle as the mouse moved past the center line. An aluminum box surrounded the cage to provide uniform enclosure conditions. Fresh air was supplied to the enclosure through 2 symmetrically placed manifolds with many small ports so that an adequate flow could be maintained without a strong blast. One-half of the enclosure was under 8 inches of lead which reduced radiation to less than 10^{-3} of that in the unshielded half. Shield and exposure cage were enclosed in a light-tight box to eliminate external visual clues to the test animal. An electrical contact closed when the mouse was in the exposure half of the cage. This contact actuated a linear potentiometer driven by a synchronous motor to put a steadily increasing signal onto a strip chart recorder. In a symmetrical environment the normal expectation would be for 50% occupancy in either half of the exposure cage. When the animal was in position voltage supplied to the potentiometer was made twice the calibration voltage. Fifty per cent occupancy would then produce a record having segments with twice the calibration slope interspersed with segments of zero slope. Thus a 50% occupancy record would follow in staircase fashion the smooth calibration line. Adult mice of NIH strain were used. Before each run a calibration was made to establish the 50% occupancy slope. Then a mouse was put in the test situation for a run without radiation. Recording started 5 minutes after the light-tight box was closed and continued 30 minutes. During this control all machinery capable of providing auditory clues was put in operation. A random orientation around the center line of the vertical beam was used to randomize unsuspected auditory clues. After each run the plastic box was thoroughly washed in detergent. If this step was omitted the next occupant would not divide his time equally between the two halves of the box. Five days after the control run each mouse was run with radiation at one of the nominal dose rates of 50, 100, 170 or 200 r/minute with 10 animals in each dose rate group. All radiations were done with X-rays produced at 3 Mev with a Van de Graaff generator, at one meter TSD.



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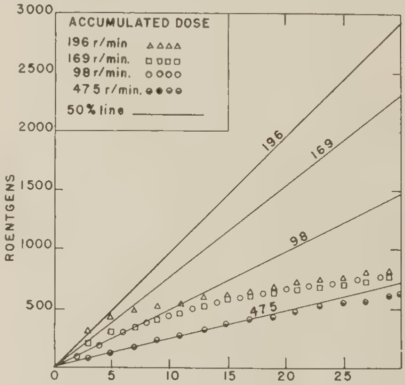

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FIG. 1. Records of occupancy of test cage, with and without radiation. Coordinate lines on recording paper have been lost in reproduction.

FIG. 2. Mean dose accumulation at the 4 dose rates.

Results. Although there were individual differences each animal was sufficiently active to sample each half of his environment a good many times during a 30 minute run. The records shown in Fig. 1 are typical. With no radiation each animal followed the 50% line quite closely, indicating a lack of bias in the test environment.

With radiation the 50% line was followed for a few minutes and then each animal began to show a preference for the shielded section. Each venture outside was short, hence the total stay outside was below normal expectancy. Although the number of in-out cycles was reduced in the presence of radiation there were enough trials in each case to suggest that the animal recognized the outside environment and found it unpleasant.

By the time an animal had established a preference for the shielded environment he had received a substantial dose of radiation, and this may have been a factor in reducing the activity(3). Thus in Fig. 1 the 98 r/minute animal showed a definite deviation from normal expectancy at 9 minutes and 440 r while the 200 r/minute animal made a choice at 2 minutes and 200 r.

Fig. 2 shows mean normal expectancy, control occupancy, and accumulated radiation dose for the 4 dose rates. There was, of course, no actual dose accumulation for controls, the values showing what would have been received if the beam had been on. In each group there is a definite radiation avoidance, starting after a dose of 300-400 r has been received.

During the last 10 minutes each group accumulated radiation at the rate of about 15 r/minute, independent of dose rate available. The 30 minute dose accumulations are surprisingly similar, being 625, 810, 780 and 830 r respectively.

Discussion. Relatively large variations might be anticipated in experiments involving animal behavior and in general the present data are surprisingly consistent. With no radiation, occupancy of the test cage was essentially symmetrical indicating a nearly clueless environment. At an available dose rate of 47.5 r/minute there was increased variability in individual responses, some animals spending more than expected time in the beam. Response patterns suggest an awareness of a new environmental element without recognizing it as unpleasant or threatening.

At 98 r/minute and above the new element is increasingly recognized as unpleasant and reaction to it becomes more uniform. At 200 r/minute the irradiated area was avoided for relatively long periods and ventures into the beam were short.

The present experiments are not adequate to establish whether avoidance arises from a direct stimulation or is the result of stimulation by radiation products such as ozone or the oxides of nitrogen. An argument against a secondary stimulation lies in the relatively constant dose, but variable time, at which avoidance becomes established. Under the experimental conditions equilibrium concentrations of gaseous radiation products should be attained very rapidly and recognition should be equally prompt.

It appears that the clue to avoidance does not lie in the emission of visible light arising from fluorescence in the irradiated plastics. Control experiments were run with the enclosure uniformly illuminated at a level high enough to mask any feeble emissions due to fluorescence. Avoidance behavior was identical with that seen in darkness.

The fact that avoidance is established at about the same dose as in the rat(2) is interesting but probably fortuitous.

Summary. Ability of mice to recognize presence of X-ray beam, and to consider it obnoxious, has been investigated. At 50 r/minute and above there is an increasing avoidance of the radiation beam. Avoidance may be due to presence of ozone and oxides of nitrogen, although this seems unlikely from results of controlled ventilation experiments. Direct visual clues from fluorescence in the plastic cage are not responsible for the avoidance.

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Production of Lesions in Guinea Pig Brain by Radon Seeds. (25598)

William V. Trowbridge and Earl Eldred

Dept. of Anatomy, School of Medicine, University of California at Los Angeles, and the Veterans Administration Hospital, Long Beach

Radioactive seeds have been used for experimental production of lesions deep within the central nervous system in dogs(1,2,3) and monkeys(4). The present work deals with the feasibility of extending this technic to a smaller animal, the guinea pig, and in particular with the tolerance of this animal to implantation of gold and glass radon seeds in the brain.

Method. Single implantations were made with glass-encapsulated radon seeds in 61, and with gold-shielded seeds in 17 adult guinea pigs. Under ether anesthesia and sterile conditions, a small hole was bored in the calvarium, and the dura was incised at a site which permitted placement of the seed in the center of one hemisphere, 5 mm back of the frontal pole. The seed was expressed from the tip of a lumbar puncture needle by insertion of a stylet, the needle being withdrawn 3 mm from its maximum depth to form a short channel. Glass seeds were 2 to 4 mm in length and 0.5 mm in external diameter, with walls of 0.1 mm thickness. Gold seeds had walls 0.25 mm thick, a diameter of 0.7 mm and a length of 2-5 mm. Strengths of seeds as measured initially by the commercial supplier* ranged to 7.0 mc for glass seeds and 12.0 mc for gold ones. Each seed was used in several animals, strengths at implantation being calculated from initial values on the basis of a half-life for radon of 3.8 days. No examination of the emission was made, but the values are probably similar to those given by others showing that about 90% of total radiant energy emitted by gold radon seeds is gamma radiation and the rest is beta emanation, whereas 96% of the emission from glass seeds are beta rays(5). Per mc of radon total radiant energy from a gold seed is roughly only 3% of that of a glass one. Calculation from these values indicates that a gold seed of 1.5 mc emits approximately the same amount of gamma radiation as a 1.0 mc glass seed.

Fatalities were noted to the nearest 12-hour period and survivors sacrificed at one week. Lesions were measured at the equator of the seed emplacement on Weil-stained sections.

Results. All but one animal which had received glass seeds of more than 1.5 mc died within 2 days, and about half of those animals in which seeds of approximately 1 mc were placed died within one week (Fig. 1). Seventeen of 18 guinea pigs with glass seeds having less than 0.75 mc survived beyond 6 days. Deaths were particularly plentiful during the 6 to 18 hours after implantation, and 23 animals died during this period. These deaths cannot be attributed to causes unrelated to irradiation injury, since none of 23 guinea pigs which received seeds of less than 0.75 mc died in this early period.

Only 3 of 17 animals receiving gold seeds were alive at one week, the seeds in these 2 having initial values of about 4 mc (Fig. 2). Deaths were more scattered in time than following glass-seed implantation and, though animals with stronger seeds tended to die within 2 days, only 2 guinea pigs died at the 12-hour period.

Discussion. The basic advantage afforded by radioactive seeds in making experimental lesions in the brain lies in the gentle and gradual nature of the destruction, which permits functional compensations vital to the animal to be established. Ability of the animal to endure the seeds will, of course, depend on site of placement. The dog may, for example, survive implantation of glass seeds of 1 mc if the site is in the cerebrum(4), while in the medulla bilateral insertion of seeds exceeding 0.015 mc (each) will bring on death (3). In the guinea pig, cerebral implantations show that doses produced by glass seeds of more than 1 mc are not tolerated, and for insurance of survival beyond one week the seed strength should be reduced below 0.5 mc. In this respect it may be noted that, in terms of general susceptibility to ionizing irradiation,

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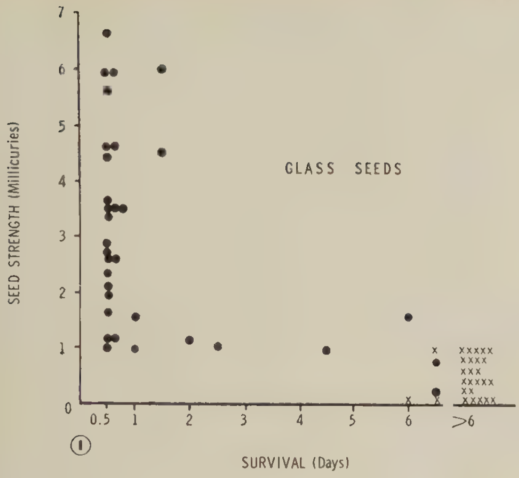
* Henry N. Beets Co., Los Angeles, Calif.



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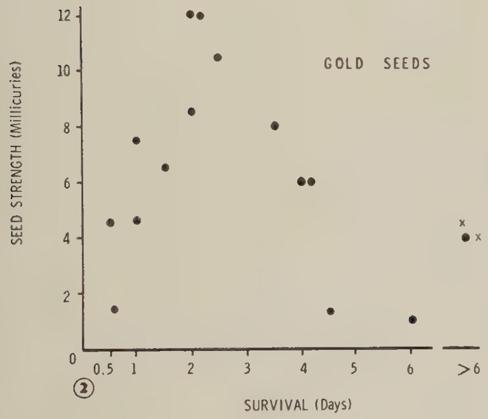

②

FIG. 1 and 2. Graphs relating survival in days to initial strength in millicuries of glass- and of gold-shielded radon seeds. Dots designate spontaneous deaths and crosses represent sacrificial deaths.

the guinea pig is probably no more sensitive (MLD_{30} is 400 to 500 r(6,7)) than other laboratory animals.

Patterns of survival under implantation with the 2 types of seeds differ in the high incidence of deaths with glass seeds within the first 18 hours and absence of such early lethality with gold seeds. This contrast is probably related to the nature of the radiations. Glass seeds emit poorly penetrating beta rays which produce an intense lesion concentrated about the seed. The early deaths primarily result from this beta radiation and not the associated gamma rays, judging from the fact that gold seeds having gamma emission equal to the gamma output of even the strongest glass seeds used do not cause early death. Emanation from gold seeds on the other hand is comprised largely of gamma rays which may produce more diffuse injury and at greater distances, including in these small brains vital brain stem centers. Although the few observations on gold seeds of low strength do not permit delineation of the threshold for 6-day survival with gold seeds, the fact that most animals survived with glass seeds of 1 mc or less indicates that gold seeds of equal gamma emission, that is, 1.5 mc and less, should not produce death. This value is a minimal one as the threshold strength for the glass seeds is presumably dependent upon the beta radiation, and, in fact, survival of several animals with 4 mc gold seeds points to a median lethal threshold at about that strength. Neither the early deaths with glass seeds nor the delayed ones with gold seeds, incidentally, correspond necessarily to deaths in dogs following implantation in the medulla, for these occurred only after 6 days(3).

Glass seeds of strengths permitting survival (0.4 to 0.6 mc) were found to produce lesions up to 5 mm in diameter in one week. Only minor enlargement of the lesion is to be expected at longer periods as the effective half-life of a radon seed is 3.8 days. Borison and Wang, for example, found that 80% of ultimate size of the lesion was attained by 7 days (3). Thus, it appears that glass seeds can be used to make rather extensive lesions in animals which will survive for at least one week. All animals with gold-seed induced lesions of significant size, however, died within the first week.

Summary. Gold and glass radon seeds of varying strength were inserted into one cerebral hemisphere of mature guinea pigs and duration of survival up to one week observed. Animals having glass seeds of greater initial strength than 1.5 mc died within 2 days, whereas those with seeds of less than 0.7 mc survived. As glass seeds of about 0.5 mc produce lesions several millimeters in diameter, they may be used for short-term experimental production of lesions in this small animal. Gold seeds, however, cannot be used for this purpose.

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Comparative Passage of Sr^{85} and Ca^{45} from Plasma into Body Fluids in Man.* (25599)

Joseph Samachson, Bernard Kabakow,† and Herta Spencer
(Introduced by B. A. Sachs)
Division of Neoplastic Diseases, Montefiore Hospital, N. Y. City

In vitro studies on comparative ultrafiltration of strontium and calcium(1) have shown that a lower percentage of strontium than of calcium is bound to plasma proteins. However, the passage of the 2 elements from plasma into cerebrospinal fluid in man occurred at similar rates(2), indicating that the membranes through which the ions pass discriminate in favor of calcium. In an extension of these investigations, transfer of calcium and strontium across membranes of other body cavities was studied and passage of both ions from plasma into peritoneal, pleural and interstitial fluid was investigated.

Materials and methods. Transfer of either Sr^{85} alone or of Sr^{85} and Ca^{45} from plasma into various body fluids was studied in 9 patients. The diagnoses of the patients and types of studies performed are listed in Table I. In patients with ascites in whom a paracentesis was indicated, a polyethylene catheter was inserted into the peritoneal cavity through a No. 14 needle after anesthetizing the skin. Ascitic fluid was obtained for analyses of protein and calcium by aspiration from the polyethylene catheter, the open end of the tubing being clamped afterwards. A tracer dose of either Sr^{85} or of Sr^{85} and Ca^{45} as the chloride was injected intravenously and samples of plasma and peritoneal fluid were then obtained serially. The patients were kept on a slowly oscillating bed for 4-8 hours in an attempt to obtain thorough mixing of peritoneal fluid with the radioisotopes. The peritoneal fluid was withdrawn 24 hours after injection of the tracers, to measure the fluid volume. Transfer of calcium and strontium from peritoneal fluid into the blood stream was studied in one patient by injecting the tracers into the peritoneal cavity and obtaining samples of ascites and plasma as indicated above. Transfer of the 2 elements from plasma into pleural fluid was also studied in 2 patients in a similar manner. Comparative concentration of radiocalcium and radiostrontium in plasma and interstitial fluid was studied in a patient with massive edema of the lower extremities (Patient 5) following intravenous injection of the tracers. Interstitial fluid was obtained by Southey tubes inserted into the subcutaneous tissue. Samples of plasma, peritoneal, pleural and interstitial fluid were analyzed for Sr^{85}, Ca^{45}, calcium, phosphorus and total protein. Sr^{85} was counted in a well-type scintillation counter and Ca^{45} was counted after precipitation with carrier calcium as the oxalate in a gas flow counter(3). Stable calcium was determined by the method of Kramer and Tisdall as modified by Clark and Collip(4), inorganic phosphorus by the method of Fiske and SubbaRow (5), and protein in serum and fluid by the Kjeldahl method, a small correction being made for non-protein nitrogen. Rates of flow

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† Post-doctoral Research Fellow, Nat. Found., 1957-1958.

TABLE I. Nine Patients Studied.

Age, sex	Diagnosis	Studies performed
76, ♀	Carcinoma of ovary	Sr^{85} transfer into ascites fluid
53, ♀	*Idem*	*Idem*
61, ♀	Carcinoma of breast	$Sr^{85} + Ca^{45}$ transfer into ascites fluid
55, ♀	" of ovary	*Idem*
51, ♂	Cirrhosis of liver	"
60, ♂	*Idem*	" *
36, ♀	Hodgkin's disease	$Sr^{85} + Ca^{45}$ transfer into interstit. fluid
54, ♀	Carcinoma of breast	$Sr^{85} + Ca^{45}$ transfer into pleural fluid
51, ♀	Rheumatic heart disease, cirrhosis of liver	*Idem*

Tracer doses of Sr^{85} and/or Ca^{45} were inj. intrav. to all patients.

* Patient 6 received, in addition to intrav. dose, an intra-abdominal inj. of $Sr^{85} + Ca^{45}$.

of fluid from plasma into the peritoneal cavity were calculated on the assumption that amount of radioisotope passing from the vascular space into the peritoneal cavity was proportional to the difference of its concentration in plasma and ascites at any time, no allowance being made for binding of the isotopes to protein. Rates were calculated for each collection period from this difference of the isotope concentration. If R is rate of passage of fluid into and out of the peritoneal cavity in ml/min, t time in minutes, V total volume of peritoneal fluid in ml, assumed to be constant, $\bar{P}$ and $\bar{F}$ are average radioisotope concentrations in plasma and peritoneal fluid, expressed in percent of dose/ml, respectively, and ΔF is increase of isotope concentration in peritoneal fluid during the period in question, increase of radioisotope in the peritoneal fluid in a given period is $V\,\Delta\,F = Rt\,(\bar{P} - \bar{F})$, and $R = \dfrac{V\,\Delta\,F}{t\,(\bar{P} - \bar{F})}$.

Ultrafiltration studies were carried out on plasma and peritoneal fluid of Patient 6 at 37°C by a modification of the method of McFarland and Hein(6,7), as previously described(1).

Results. Table II presents results obtained on passage of radioactive tracers from plasma into peritoneal fluid of 2 patients with carcinomatous effusions. In Patient 3, rate of flow calculated from Sr^{85} data differed from rate calculated from Ca^{45} data, Sr^{85} entering the peritoneal fluid at approximately half the rate of Ca^{45}, while in Patient 4, both radioisotopes entered at approximately the same rate. Plasma levels for Sr^{85} and Ca^{45} were identical within limits of experimental error in Patient 3, while plasma Ca^{45} values of Patient 4 were initially lower than those of Sr^{85}, reaching approximate equality at 24 hours.

Table III shows data obtained on a patient with cirrhosis of the liver. An intravenous injection of Sr^{85} alone was first given followed by an intravenous injection of Sr^{85} and Ca^{45} 3 months later. Sr^{85} plasma and ascites levels of these 2 studies agreed well. Sr^{85} and Ca^{45} data obtained in the second study (Table III) indicate a slightly greater rate of passage of Sr^{85} than of Ca^{45} from plasma into ascites, comparative rate of passage of Sr^{85}/Ca^{45} being 1.14/1.03 or 1:1. Ultrafiltration studies carried out on plasma and peritoneal fluid of this patient revealed that 61% Sr^{85} and 51% Ca^{45} were ultrafilterable from plasma (ratio 1.2) and 76% Sr^{85} and 67% Ca^{45} from peritoneal fluid at 37°C. The lower half of Table III presents data on passage of the radioisotopes from peritoneal fluid into plasma following an intracavitary injection of both radioisotopes. Both tracers

TABLE II. Passage of Sr^{85} and Ca^{45} from Plasma into Peritoneal Fluid Following Intravenous Injection of Tracers in Patients with Carcinomatous Effusions.

Time (hr)	Plasma, % dose/l		Plasma	Peritoneal fluid, % dose/l		Ascites
	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}
			Patient 3			
⅓	4.54	4.54	1.00	.10		
⅔	3.19	3.30	.97	.12	.20	.60
1½	2.44	2.43	1.01	.22	.40	.55
4	1.65	1.57	1.05	.37	.65	.57
8	1.26	1.19	1.06	.50	.89	.56
24	.72	.64	1.13	.70	1.13	.62
		Avg	1.04		Avg	.58
			Patient 4			
½	7.02	6.11	1.15	.81	.66	1.23
1	5.64	4.84	1.17	1.08	.95	1.14
2	4.59	4.02	1.14	1.55	1.36	1.14
3	4.02	3.49	1.15	1.89	1.63	1.16
4	3.73	3.11	1.20	2.08	1.86	1.12
8	3.11	2.73	1.14	2.60	2.27	1.15
24	2.37	2.17	1.10	2.44	2.23	1.09
		Avg	1.15		Avg	1.15

TABLE III. Passage of Sr^{85} and Ca^{45} between Plasma and Peritoneal Fluid Following Intravenous and Intracavitary Injection of Tracers in Patient with Cirrhosis of Liver.
Patient 6

Time (hr)	Plasma, % dose/l		Plasma	Peritoneal fluid, % dose/l		Ascites
	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}
Intrav. inj. of Sr^{85} and Ca^{45}						
¼	5.98	5.85	1.02	.17	.17	1.00
½	4.99	4.92	1.01	.33	.27	1.22
1	4.32	3.94	1.10	.47	.40	1.18
4	2.68	2.56	1.05	1.03	.86	1.20
8	2.11	2.00	1.06	1.35	1.16	1.16
24	1.48	1.56	.95	1.32	1.17	1.13
		Avg	1.03		Avg	1.14
Intracav. inj. of Sr^{85} and Ca^{45}						
⅓	.13	.11	1.18	8.29	8.13	1.02
½	.27	.26	1.04	7.71	7.69	1.00
1	.45	.43	1.05	7.54	7.37	1.02
4	1.06	1.02	1.04	5.82	5.74	1.01
6	1.33	1.19	1.12	5.18	4.82	1.07
7½	1.42	1.29	1.10	4.40	4.13	1.07
11	1.48	1.37	1.08	3.45	3.18	1.08
12½	1.53	1.33	1.15	3.02	2.86	1.06
15	1.52	1.35	1.13	2.48	2.30	1.08
24	1.46	1.34	1.10	1.62	1.36	1.19
		Avg	1.10		Avg	1.06

passed into the plasma at approximately the same rate.

Table IV lists data on passage of the radioisotopes from plasma into interstitial fluid in a patient with Hodgkin's disease and marked edema of the lower extremities. Concentrations of Sr^{85} and Ca^{45} in plasma were almost identical for 4 days and in interstitial fluid for 5 days of the study. Sr^{85} and Ca^{45} values in the interstitial fluid appeared to reach a maximum at about 4 hours, indicating a time lag in passage of approximately the same order for both radioisotopes. The edema fluid contained 2.8 g % protein and 10.3 mg % Ca, compared with 6.2 g % and 13.6 mg% respectively in the plasma.

TABLE IV. Passage of Sr^{85} and Ca^{45} from Plasma into Interstitial Fluid Following Intravenous Injection of Tracers in Patient with Hodgkin's Disease.
Patient 7

Time (hr)	Plasma, % dose/l		Plasma	Interstitial fluid, % dose/l		Interstitial fluid
	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}
1¼	3.23	2.76	1.17	1.37	1.34	1.02
4	2.16	2.09	1.03	1.68	1.59	1.06
21	1.53	1.52	1.01	1.28	1.34	.96
24	1.35	1.39	.97	1.22	1.27	.96
92	.97	.92	1.05			
120				.69	.70	.99
		Avg	1.05		Avg	1.00

Table V lists rates of passage of fluid between plasma and peritoneal cavity calculated from Sr^{85} and Ca^{45} data. Rates of flow calculated from Sr^{85} in 6 patients ranged from 3 to 28 ml/min. There was good agreement of rates of flow in Studies I and II performed in Patient 6, while there was a 2-fold increase in Study III. The ratio of Sr^{85}/Ca^{45} was approximately 1:1 in Patient 4 and in Study III of Patient 6 and possibly also in Study II of the same patient. The ratio was approximately 1:2 in Patient 3. There was no apparent correlation between rate of flow and protein concentration in plasma or ascites. The net increase in volume of peritoneal fluid over the period studied as determined clinically was relatively negligible, so that rates of passage into and out of the peritoneal cavity could be assumed to be approximately the same.

TABLE V. Rate of Passage of Fluid between Plasma and Peritoneal Fluid Calculated from Sr^{85} and Ca^{45} Data. Intravenous injection of tracers.

Patient	Ascites vol, ml* (× 100)	Rate of flow, ml/min., from data		Total protein, g %	
		Sr^{85}	Ca^{45}	Plasma	Ascites
1	70	20		6.6	2.2
2	85	28		6.6	5.5
3	50	3	7	6.5	4.5
4	45	10	10	7.3	4.9
5	47	8		7.6	1.0
6 Study I	80	10		6.2	1.7
II	70	10	8	6.1	1.4
III	110	24†	24†	5.9	1.8

* Removed by paracentesis after completion of the study.

† The tracer was inj. into the peritoneal cavity.

The ratios of pleural fluid/plasma Sr^{85} and Ca^{45} of 2 patients listed in Table VI indicate greater passage of Sr^{85} than of Ca^{45} into the pleural cavity although pleural fluid protein of Patient 8 was high (this patient had a carcinomatous effusion) and pleural fluid calcium of both patients was almost as high as that of plasma. Rates of passage of both Sr^{85} and Ca^{45} into the pleural fluid were higher in Patient 9 than in Patient 8.

Discussion. Concentrations of protein and calcium indicated that none of the body fluids studied were genuine ultrafiltrates. Formation of abnormal body fluids is obscure. It has been suggested that the intrinsic capillaries of the liver are extremely permeable and produce hepatic lymph, high in protein, which may be a possible source of ascitic fluid(8). However, this process of ascitic fluid formation may not be applicable to effusions resulting from various etiologies. The process is all the more obscure because flow of fluid is in both directions. The net rate of formation of ascitic fluid was estimated to be less than 0.5 liter/day, *i.e.*, less than 0.4 ml/minute the slowest rate of passage calculated from the Sr^{85} data being 3 ml/minute (Table V). When tritium-labeled water is used, rate of flow is found to be higher, so that 40-80% of the total ascitic fluid volume enters and leaves the peritoneal cavity per hour(9). This rate of flow would correspond to a passage of 58 to 115 liters of water from plasma into the abdominal cavity within 24 hours in a patient with 6 liters of ascites. In studies carried out with radio-labeled albumin, complete albumin turnover in the fluid has been reported to occur in 2 days(10), whether albumin was injected intravenously or intraperitoneally. Comparing the low rates of passage of fluid derived from Sr^{85} and Ca^{45} data with those obtained with tritium, one may conclude that the 2 ions do not diffuse as freely into and out of the peritoneal fluid as water but may enter the fluid in part combined with protein and in part unbound.

Although ascitic fluid is not an ultrafiltrate, relative Sr^{85}/Ca^{45} values in plasma and ascites of Patient 6 following intravenous and intracavitary injection of the tracers were similar to those found in ultrafiltration experiments (Table III). Studies carried out in 2 patients with carcinomatous effusions revealed a ratio for passage of fluid from plasma into the peritoneal cavity of 3:7 and 1:1 when calculated from Sr^{85} and Ca^{45} data, respectively, (Table V), indicating no preferential passage of Sr^{85}. Rate of flow from plasma into peritoneal fluid varied considerably from patient to patient. In Patient 6, rate of flow following intracavitary injection was much greater than after intravenous administration of the isotopes. This difference may be due to a change in status of the patient during the 3-month interval between studies. The increased volume of fluid in this patient, as well as deterioration of his clinical condition, also indicated that such a change occurred. The extremely low rate of passage in Patient 3 may have been due to dehydration. From the variable amounts of protein in different peritoneal fluids, from the different rates of passage of the isotopes and the different Sr^{85}/Ca^{45} ratios, it may be inferred that many factors affect ascitic fluid formation and that the mechanism of formation may differ from case to case.

TABLE VI. Passage of Sr^{85} and Ca^{45} from Plasma into Pleural Fluid.

	Plasma		Pleural fluid		Fluid/ plasma	
Patient	Ca, mg %	Protein, g %	Ca, mg %	Protein, g %	Sr^{85}	Ca^{45}
8	9.9	6.5	8.1	3.3	.67*	.55*
9	9.6	5.8	9.4	1.0	.81†	.74†

* 24 hr after intrav. inj. of tracers.
† 27 hr *Idem*

The Sr^{85}/Ca^{45} ratio for passage into pleural fluid from plasma was almost like that of an ultrafiltrate (Table VI). This preferential passage of Sr^{85} is noteworthy since red blood cells were present in the carcinomatous pleural effusion, indicating increased permeability of the capillaries, and both radioisotopes might be expected to pass into the pleural fluid at about the same rate. Of interest are the high values for calcium in the pleural fluid, whether the fluid was an exudate or transudate. This is contrary to what might be expected from the literature(11) but has been confirmed in our laboratory on a larger number of patients. It should be noted that a single reading of the serum calcium value may be far from representative of the patient's true calcium level since considerable fluctuations in serum calcium have been found even in healthy individuals(12) and greater fluctuations may be expected to occur in pathologic states.

Summary. The passage of strontium and calcium from plasma into peritoneal, pleural and interstitial fluids has been studied in man following intravenous injection of Sr^{85} and Ca^{45}. Rates of passage of the 2 isotopes into peritoneal cavity have been calculated. In 1 case, Ca^{45} entered the cavity more rap-

idly than Sr^{85}, in another, Sr^{85} entered more rapidly, and in 2 cases, both radioisotopes entered at the same rate. Both ions passed more rapidly into and out of the peritoneal cavity than the fluid accumulated, but passage of ions was slower than flow of water. Rate of passage varied in different patients as did ratio of rates for Sr^{85} and Ca^{45}. The high protein content indicated that peritoneal fluid was not an ultrafiltrate although the Sr^{85}/Ca^{45} ratio corresponded to that of an ultrafiltrate in some cases. The ratio in pleural fluid approximated that of an ultrafiltrate, Sr^{85} entering more rapidly. The ratio of passage of Sr^{85} and Ca^{45} into interstitial fluid studied in one patient with massive edema indicated increased permeability of the capillaries, both isotopes entering the fluid at about the same rate.

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Lipid Bound Glutamic Acid Deficiency in Aging Arteriosclerotic Subjects.*†
(25600)

R. E. HAMILTON AND L. O. PILGERAM
St. Barnabas Hosp. Research Foundation and Dept. of Physiology, University of Minnesota School of Medicine, Minneapolis

A previous report(1) described chromatographic identification of bound glutamic acid in the lipid fraction of rat liver. Our object was to determine whether this factor also exists in human plasma lipoproteins and whether the compound shows variation in the aging arteriosclerotic human subject.

Methods. Twenty ml of blood were introduced into a centrifuge tube containing 1.5 ml acid citrate dextrose solution. Fractionation of alpha and beta lipoproteins was carried out according to method of Lever *et al.*(3). Following fractionation, the tubes containing alpha- and beta-lipoproteins received 75 ml of ethyl alcohol:ethyl ether (3:1) extraction mixture. The suspension was stirred 3 hours at room temperature. Then the clear supernatant was decanted into 125 ml Erlenmeyer flasks. To the precipitate were added another 75 ml of lipid extracting media, suspension stirred, then stored overnight at –25°C(4). After removal from deep freeze, stirring was maintained 3 hours. The tubes were then centrifuged and this second extraction was pooled with the previous one. This extraction procedure was repeated using anhydrous ethyl ether. Pooled extracts were brought to anhydrous state over sodium hydroxide pellets *in vacuo* at 40°C. Phospholipids were extracted from the residue with three 15 ml portions of anhydrous petroleum-ether (b.p. 30°-60°C). If residue and solvent are anhydrous, there is no contamination from amino acids

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which may have been absorbed to initial precipitate and carried over in alcohol-ether extraction(5). Pooled petroleum-ether extracts were evaporated from reflux flasks at 65°C. The lipids were hydrolyzed with 25 ml of 6 N HCl at 108°C for 22 hours. After chilling, the hydrolysate was quantitatively filtered to remove insoluble fatty acids. After filtration and drying *in vacuo* at 45°C-50°C, the residue was taken into solution with 10 ml of warm, distilled water and hydrolysis products were separated on a Dowex-50 (400 mesh) cation exchange column (0.9 x 55 cm). The flow was adjusted to 7 ml/hour and 10 ml samples of 1.5 N HCl eluate were collected. Glutamic acid and serine were eluted in the vicinity of 90 ml of eluate. All collection vials were dried *in vacuo* with inside desiccator temperature of 40°C. Glutamic acid and serine were separated *via* descending paper chromatography using a solvent system of butanol-acetic acid-water (21:50:100) for 30 hours. It is not necessary to precede paper chromatography with column chromatography. It was used here because of simultaneous need for adequate amounts of phospholipid bases for other analyses not concerned with this report. After the paper was removed from chromatography chamber and dried at room temperature for at least 3 hours, it was rapidly dipped in a methanolic-ether-potassium hydroxide solution (3 parts of methanol, 1 part ether, 1% KOH), which removed an erratic background during color development. After drying, the paper was dipped in 0.1% ninhydrin acetone solution (pH 5) and dried at room temperature 30 minutes. Amino acid spots become visible but instead of both glutamic acid and serine giving typical blue-purple spot, serine becomes straw-colored whereas glutamic acid becomes pale blue. Equal areas were cut from the paper, including blanks as well as amino acids, and placed into flat bottom vials. One ml (pH 5) citrate buffer, 0.05 ml of 0.7 N acetic acid, and 1.5 ml of 2% ninhydrin solution (pH 5) were added. After 5 minutes shaking, vials were placed in covered, vigorously boiling water bath for 25 minutes. After chilling to room temperature the colored solutions were transferred to 10 ml volumetric flasks. Two-3 ml portions of propanol-water diluent were used to extract all color. Rinses were pooled into flasks; the flasks diluted to proper volume with diluent, mixed, and read at 570 mμ. Standards were run with each analysis. *Minimum requirements* for accepting a patient as an arteriosclerotic subject were (a) clinical and laboratory history of myocardial infarction from which the subject had recovered for at least 4 months, and (b) electrocardiographic changes characteristic of an infarction. Plasma was drawn only from subjects who fasted a minimum of 8 hours overnight prior to taking a sample. *Recovery.* One hundred or more μg of glutamic acid introduced into the analytical procedure beginning with acid hydrolysis, provided individual recovery values of 96, 107 and 100%. Serine, choline, and ethanolamine were also determined and gave recoveries of 96 ± 6%, 97 ± 4%, and 106 ± 6% respectively. Recovery of glutamic acid or serine in amounts less than or equivalent to 50 μg was low, ranging from 48 to 83%. No adequate explanation appears for this low recovery, although it is possible that some glutamic acid may have been converted to pyrrolidone carboxylic acid. The method did not extract free glutamic acid (1).

Results. The results of this study are given in Table I. It is apparent that the lipid bound, ninhydrin positive constituent identified as glutamic acid is present as a constituent of human plasma and that this new lipid factor is deficient in the aging, arteriosclerotic human subject. The average normal subject has 33.9 μM per liter of plasma as compared to 17.0 μM for the average arteriosclerotic. The change in distribution ratio of glutamic acid between alpha and beta lipoprotein fractions favors the beta fraction and appears to be a part of a defective mechanism for metabolism of these lipoprotein moieties. The normal subject has 47% in alpha fraction compared to 35% in this fraction for the arteriosclerotic. This abnormal distribution ratio correlates with the change in alpha-beta distribution ratio of cholesterol as observed by Barr *et al.*(6) in arteriosclerotic patients. In passing from the normal to the arteriosclerotic condition, alpha lipoprotein incurred, on the average, a 63% loss ($p<0.003$) in this lipid factor. The arteriosclerotic beta lipo-

TABLE I. Lipid Bound Glutamic Acid in Normal and Arteriosclerotic Subjects.

Normal subjects	H.K.	S.G.	D.S.	I.G.	G.S.	O.H.
Age	44	18	18	21	26	35
Wt	205	140	140	180	165	155
Alpha fraction	.394*	.200	.238	.164	.232	.182
Beta "	.332	.374	.172	.174	.346	.186
Total	.726	.574	.410	.338	.578	.368

Arteriosclerotic subjects	A.T. &	W.L.	W.F. &	P.A.	G.M.	W.M.	E.D.
Age	44	51	63	70	62	50	54
Wt	225	206	—	185	164	180	180
Date of infarction	3/57	3/57	7/56	6/56	1955	5/57	5/57
Alpha fraction	.028		.040		.156	.136	.100
Beta "	.168		.080		.154	.248	.132
Total	.196		.120		.310	.384	.232

* All values reported in mg %.

protein showed about a 37% loss with respect to the normal in lipid bound glutamic acid, however, range of variability in beta lipoprotein glutamic acid was sufficiently great as to cause this value to border on being significant.

In the differential ethanol separation of Cohn's microfractionation method No. 10 the 2 major types of lipoproteins, alpha and beta, are concentrated in separate fractions. Electrophoretic determinations of plasma fractions from normal individuals and arteriosclerotics have indicated that separation of the lipoproteins is complete. It appears reasonable to assume that the lipid bound glutamic acid is carried by the alpha and beta lipoprotein. This consideration is supported by the observed change in percentage distribution of glutamic acid between alpha and beta lipoprotein in normal and pathological subjects and correlation of this change to known changes in plasma content of alpha and beta lipoproteins with arteriosclerosis(6). Our measurements can not exclude the possibility that this factor is selectively isolated with or adsorbed to the alpha and beta lipoprotein or one of the other fractions; however, observations (3,6) that lipids recovered from fraction IV + V + VI are in combination with alpha$_1$ globulin and those recovered from fraction I + II + III are combined with beta$_1$ globulin would strongly argue for specific binding to alpha and to beta lipoprotein fractions.

Hecht(2) has demonstrated a highly significant inhibitory effect of glutamic acid on thrombokinase activity in the blood coagulation system. It is not known whether the lipid bound glutamic acid factor described here possesses thrombokinase inhibitory activity. If it should possess this inhibitory capacity, this fact taken together with its striking deficiency in arteriosclerotic subjects, who possess markedly disturbed blood coagulation systems(7), should warrant further work on the structure and defective metabolic relationships of this factor.

Finally, it can not be stated whether the observed difference between normals and pathologicals represents a loss of lipid bound glutamic acid in the pathological subject or whether we are dealing with molecular structural differences which convey differential solubility properties.

Summary. A ninhydrin positive, lipid bound factor chromatographically identified as glutamic acid has been found in the organic solvent extract of human plasma alpha and beta lipoproteins. This factor is grossly deficient ($p<0.006$) in plasma of the aging, arteriosclerotic subject and has an abnormal distribution pattern between alpha and beta lipoproteins of the pathological patient. This deficiency of lipid bound glutamic acid may be related to a defective blood coagulation system of the human arteriosclerotic.

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Effect of S-(Dichlorovinyl)-L-Cysteine on Incorporation of Precursors into Deoxypentose Nucleic Acid of Leukocytes.* (25601)

N. S. MIZUNO, G. J. HAGERTY, D. D. JOEL, J. H. SAUTTER AND M. O. SCHULTZE
Division of Vet. Pathology and Parasitology, College of Vet. Med., and Dept. of Agricultural Biochemistry, University of Minnesota, St. Paul

Continuing our investigations on the mechanism by which trichloroethylene-extracted soybean oil meal or S-(dichlorovinyl)-L-cysteine (DCVC)(1) can induce fatal aplastic anemia in calves we studied the effects of DCVC on incorporation of radioactively labeled precursors into deoxypentose nucleic acid (DNA) of leukocytes.

Procedures. Experiments with P^{32}-phosphate. Male Holstein calves, weighing 100-130 lb were conditioned at least 2 weeks prior to experiments until judged normal by clinical and hematological observations. Sodium P^{32}-phosphate, (2.5 mc, specific activity: 48-208 c/g, pH = 7.4, in 0.9% aqueous sodium chloride) was injected intravenously. A dose of DCVC (total of 3.3 mg/kg in 3 equal portions during 10 hr), previously found to induce fatal aplastic anemia in young calves(1), was injected intravenously on same day as P^{32} (first portion 5 hr before P^{32}; 2 calves) or 2 days prior to P^{32} (4 calves), or 4 days prior to P^{32} (2 calves). Four calves receiving no DCVC served as controls. All calves were kept in metabolism cages and fed, twice daily, suspension of hexane-extracted soybean oil meal in milk. Counts of thrombocytes, total and differential leukocytes were made daily. Heparinized blood (75 ml) was collected 6 hours after P^{32} injection and then daily. It was centrifuged at 5°C and 2000 g for 30 minutes, plasma removed and recentrifuged. One ml of plasma and 0.1 ml of glycerol were placed in aluminum planchets lined with filter paper and dried under infrared lamp for measurement of radioactivity. The buffy coat was lifted out, briefly washed with cold 0.16 M sodium chloride-0.01 M sodium citrate solution and DNA isolated by the method of Kay *et al.*(2). DNA-containing precipitate was suspended in 2.5 ml of 5% trichloroacetic acid, heated at 100°C for 30 minutes, cooled, diluted to 5 ml with H_2O, and centrifuged. Aliquots of clear supernatant were dried on paperlined planchets to measure radioactivity, or used for DNA determinations(3). Specific activity of leukocyte DNA was calculated as counts/minute (CPM)/μg DNA. The collected urine was filtered, 1 ml aliquots dried in paperlined planchets under infrared lamp. Radioactivity was measured with gas-flow, thin window Geiger counter and calculated with corrections for physical decay and self-absorption.

Isolated leukocyte DNA was checked for contamination by RNA and by inorganic P^{32}. RNA was determined in trichloroacetic acid extracts by the orcinol method(4). To check for inorganic P^{32} contamination, P^{32}-phosphate was added to chilled buffy coat suspension at concentrations comparable to those found in plasma, and isolation procedure for DNA was initiated at once. Radioactivity of isolated DNA was then determined. *With tritiated thymidine.* Incorporation of this compound into leukocyte DNA was studied *in vitro* with blood from normal and DCVC-treated calves. Fresh blood containing 1/10 volume disodium ethylenediamine tetraacetic

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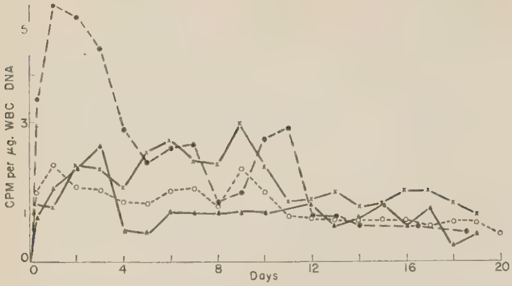

FIG. 1. Specific activity of leukocyte DNA after inj. of P^{32}-phosphate and DCVC. ● - - - - ● no DCVC (means of 4); × —— × DCVC on same day as P^{32} (means of 2); ○- - - -○ DCVC 2 days before P^{32} (means of 4); ▲——▲ DCVC 4 days before P^{32} (means of 2).

acid (1% in 0.7% aqueous sodium chloride) was placed in silicone-coated tubes, and H^3-thymidine[†] (specific activity: 360 mc/m mole) was added to final concentration of 5 μc/ml. This mixture was kept at room temperature and smears were made after 1 and 6 hours. Effects of DCVC on H^3-thymidine uptake were also studied *in vitro* by addition of DCVC to normal calf blood at concentration (1 μM/ml) which retards growth of cells in tissue culture.[‡] Autoradiographs were made with Kodak Autoradiographic Stripping Film AR-10, exposed for 6 days at 4°C, developed with Kodak D19b, fixed with Kodak acid fixer, washed and stained with Leishmann-Giemsa stain(5).

Results. Control calves had always normal blood counts. In blood of DCVC-treated calves, as observed previously(1), counts of leukocytes, thrombocytes and polymorphonuclear leukocytes remained normal for at least 12-14 days, then progressively dropped to very low levels. The DCVC-treated calves died after 20-30 days.

Fig. 1 shows that treatment of calves with lethal dose of DCVC decreased but did not abolish early incorporation of inorganic phosphate into DNA of blood leukocytes. With normal calves a maximum specific activity of about 2½ times that found with DCVC-treated calves was observed 1-2 days after administration of P^{32}. In normal calves there occurred a second, distinct rise in specific activity of leukocyte DNA between 9th and 11th day when concentration of P^{32} in blood plasma had greatly decreased, essentially to same extent as in DCVC-treated calves. No evidence was found for this in calves treated with DCVC 4 days before administration of P^{32}. Leukocyte DNA was obtained from a mixed population of cells in which lymphocytes predominated(1); no information is available on rate of DNA synthesis in different cell types or their precursors in the calf.

Radioactivity of DNA extracts was not the result of contamination with inorganic phosphate. When P^{32}-phosphate was added to a suspension of buffy coat from normal calves and DNA isolation started immediately, radioactivity recovered in DNA extract was 0.12% of the added P^{32}. Actual contamination of DNA with inorganic P^{32} in *in vivo* experiments is probably less than this; however it is not known at what stage of isolation procedure incorporation of P^{32} into leukocyte DNA stops completely. When DNA extracts were treated with orcinol reagent, colored products were obtained which, when calculated as pentose nucleic acid (PNA), gave results indicating that PNA was only about 1% of DNA present. Inasmuch as authentic DNA also gives an orcinol reaction of this order of magnitude(4), it is assumed that PNA contamination of DNA extracts was negligible.

Calves treated with DCVC 2 or 4 days before injection with P^{32}, had during the first 2 days a higher rate of urinary P^{32} excretion. This may reflect some kidney damage which becomes severe and results in high blood urea nitrogen values when higher doses (4-5 mg/kg) of DCVC are injected.[‡] In these experiments blood urea nitrogen remained normal.

When blood from normal calves was incubated with H^3-thymidine, about 1% of mononuclear cells showed nuclear uptake of the tracer, presumably into the DNA. No uptake of radioactivity was observed in polymorphonuclear leukocytes. Appreciable labeling took place in 1 hour, and corroborated the *in vivo* studies in which rapid incorporation of P^{32} into blood leukocyte DNA was observed. DCVC did not inhibit incorporation of H^3-thymidine into these cells, when collected from DCVC-treated calves (Fig. 2A) or when nor-

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[†] Purchased from Schwarz Labs., Mount Vernon, N. Y.

[‡] Our unpublished observations.

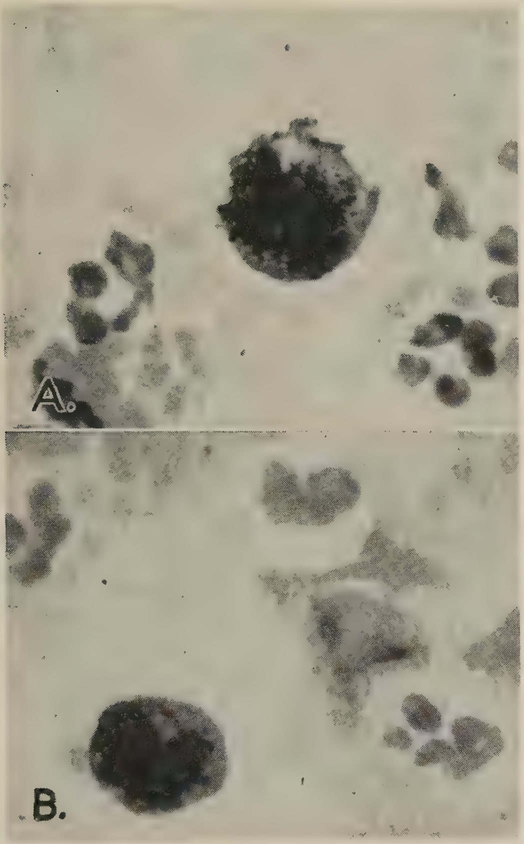

FIG. 2. Radioautographs of blood treated *in vitro* with tritiated thymidine. ×1200. A. Blood from calf treated with DCVC 18 days before collection of blood. B. Blood from normal calf incubated with DCVC (1 μmole/ml, added at same time as thymidine).

mal blood cells were incubated *in vitro* with DCVC in presence of H^3-thymidine (Fig. 2B).

Discussion. Many studies have been made with radioactively-labeled blood cells, but problems of dynamics of leukocyte production and destruction remain to be resolved(6). They are complicated through the presence of mixed populations of cells, formation of each probably being subject to independent controls. Moreover, there is evidence that leukocytes in circulation may represent only a small fraction of total leukocytes in the body, and there is little information on size and location of leukocyte pools, or on rate and extent of exchange of blood leukocytes with those in extravascular pools. Of tracer methods employed, labeling of DNA offers advantages because isotope incorporation occurs only with concurrent DNA synthesis in cells preparing for cell division, and because the label remains stably bound to DNA during lifetime of the cell; hence cells with labeled DNA represent newly formed cells. Analysis of P^{32} concentration in leukocyte DNA has been made in man(7-12), dog(13,14) and rabbit (15,16). Changes in specific activity observed in normal calves (Fig. 1) resemble the biphasic curves observed with rabbits(15,16). The fact that lymphocytes predominate in both bovine and rabbit blood may be relevant. In present studies, radioautograms were made of blood smears from P^{32}-treated calves, but the level of radioactivity was too low for satisfactory results.

Control calves showed no evidence of radiation damage, from examination of peripheral blood levels of hemoglobin, thrombocyte, leukocyte or differential counts after treatment with 2.5 mc P^{32}. This amount is less than 5 mc used for treatment of polycythemic human subjects of similar body weight(17).

When blood was incubated with H^3-thymidine, about 1% of mononuclear leukocytes showed intense labeling after 1 hour. The number of these cells did not increase appreciably after 6 hours' incubation. The majority of labeled cells were large, and resembled the mononuclear cells which Bond, Cronkite and their associates(18) suggested to be possible multipotential primitive cells with proliferative capacity, in transit in blood. Early uptake of P^{32} into DNA could be explained by presence of these leukocytes, capable of DNA synthesis while in circulation, therefore no delay would occur in appearance of labeled cells, in contrast to granulocytes(6). Either with normal blood to which DCVC had been added or with blood from DCVC-treated calves, the nuclear uptake of H^3-thymidine into these mononuclear cells was not inhibited.

Summary. 1. When sodium P^{32}-phosphate was injected intravenously into calves, there was an early labeling of leukocyte DNA. 2. DCVC decreased but did not abolish this uptake of P^{32} when given concurrently with P^{32} or when calves were pre-treated with DCVC for 2 or 4 days. 3. When normal calf blood was incubated in presence of H^3-thymidine,

there was a rapid nuclear uptake by mononuclear leukocytes. 4. DCVC did not inhibit incorporation of H^3-thymidine in either blood from DCVC-treated calves or in normal blood incubated in presence of DCVC. 5. Abolishment of DNA synthesis in leukocytes or leukopoietic tissue does not appear to be a primary biochemical lesion through which DCVC induces fatal blood dyscrasia in calves.

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Analgesia Enhancing Effect of Thalidomide. (25602)

S. C. HARRIS AND J. P. ALLGOOD
Dept. of Physiology and Pharmacology, Northwestern University Dental School, Chicago

Since it has been reported that addition of thalidomide* (N-phthalyl-glutamic acid imide) to the classical "APC" combination enhanced its clinical analgesic effect(1), it was considered interesting to study this effect experimentally on human subjects.

Method. The amount of electricity necessary to elicit a painful sensation was determined 3 times at 15 minute intervals before, and 9 times at 20 minutes after oral medication. The current was applied as square waves of 1 msec. duration every half second. The stimulus was increased by 0.01 volt at every impulse until the subjects indicated feeling pain. Temporal clues during this procedure were avoided. The method has been described(2,3). Each of the 10 subjects served for 5 experimental sessions, with at least 48 hours intervening between any 2. The sequence of medication was by "latin-square" design and "double-blind" discipline prevailed. The medications, prepared to look alike were: A. 2 APC, = 7 gr. acetylsalicylic acid, 5 gr. acetophenetidin, 1 gr. caffeine; B. 25 mg thalidomide + 2 APC; C. Placebo; D. 32 mg Codeine sulfate + 2 APC; E. "Dry-run" (no capsules).

Results. For analysis, each postmedication threshold was expressed as its increment from its average premedication voltage on the same day. The average threshold increments of the 10 subjects, by interval after and type of treatment, are presented in Table I.

An analysis of variance, performed on the detailed aggregated data (of which Table I is a summary), is summarized in Table II. This analysis compares the net (or average or areas under the curve) increments following each treatment, in terms of variance attributable to subject-treatment interaction. No significant difference was found between the average

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* "Kevadon", Trademark of Wm. S. Merrell Co.

TABLE I. Average Increments of Experimental Pain Thresholds of 10 Subjects (in Centivolts), by Time Postmedication and Treatment.

Min. after medication	Treatment A	B	C	D	E
20	6.0	.9	.2	3.9	.3
40	7.2	9.1	2.9	7.3	1.1
60	5.7	12.0	2.6	10.2	1.2
80	8.4	10.9	2.8	10.8	1.4
100	6.7	7.0	4.6	8.2	−.2
120	5.1	8.2	2.0	8.7	−.1
140	3.5	3.5	3.3	6.9	.7
160	2.3	4.6	1.5	5.0	1.2
180	−.1	3.0	.6	2.9	1.8
Total	44.8	59.2	20.5	63.9	7.4

postmedication increments which followed the control treatments C and E.

No significant difference was found between the average increments which followed the 3 treatments: A, B, and D. However, increments of the 3 treatments (A, B, and D) were significantly ($P<.01$) greater than the controls (C and E).

TABLE II. Summary of Analysis of Variance.

Source	Degrees freedom	Mean square	"F" ratio
Subjects	9	333.39	2.22*
Treatments	(4)	(667.33)	4.44†
Controls (C, E)	1	95.34	.63
Treats. (A, B, D)	2	110.05	.73
Controls *vs* treats.	1	2353.86	15.67†
Subj. × treats.	36	150.20	2.84†
Intervals	8	168.70	5.09†
Subj. × intervals	72	33.14	.63
Treats. × "	32	36.04	.68
Residual	288	52.93	

* $P <.05$. † $P <.01$.

Since it is apparent in Table I that time-response characteristics were not the same after each treatment, a separate analysis of variance was performed on all the data available within each postmedication interval.

The results of these comparisons of particular interest here are those which relate the increments after one compound to another. The only statistically significant differences obtained ($P < .05$-$.01$) indicated that the threshold was elevated above the dry run by APC + thalidomide (B) from 40 through 120 minutes after ingestion and by APC + Codeine (D) from 60 through 140 minutes postmedication. Increments after these 2 combinations (B and D) were significantly superior to placebo (C) induced increments at the 60 and 80 minute observations.

Conclusion. Because 2 APC + 25 mg thalidomide or + 32 mg Codeine sulfate elevated the threshold to experimentally induced pain of 10 human subjects above threshold increments which followed placebos or no treatment while 2 APC alone did not, it is concluded that the analgesic potential of APC is enhanced by added compounds in the quantities employed.

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Survival of Dogs Following Section of Carotid and Vertebral Arteries.* (25603)

DONALD F. M. BUNCE† (Introduced by S. R. M. Reynolds)
Dept. of Anatomy, University of Illinois College of Medicine, Chicago

The functional effects following acute arrest of the carotid and vertebral arterial blood supply to the brain aroused the interest of several investigators during 19th and part of present century(1-7). Effects of deprivation of blood flow by these routes have been observed in animals, including horse, dog, cat, rabbit, monkey and goat(2,8,9). With few exceptions(1,2), these experiments were acute in nature, the animal either succumbing, or sacrificed soon after its termination. The

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† U.S.P.H.S. Trainee in Anatomy.

present study is concerned with some chronic effects observed in dogs in which continuity of the carotid and vertebral arteries is permanently interrupted.

Procedures. Two groups of 7 dogs each were used. All animals were approximately 1-3 years old, weighed between 12-20 kg, and were about equally divided between the sexes. Anesthesia was induced with 3% nembutal, 0.7 cc/kg, and morphine, grains 1.5. In the first group of 7 dogs, both common carotid and both vertebral arteries were severed. A longitudinal incision was made in the neck extending from about the level of fourth cervical vertebra to the superior margin of sternum. Both common carotid arteries were exposed. The subclavian arteries then were sought, and their vertebral branches isolated. Using a Meyerding retractor, each vertebral artery was lifted from surrounding tissue, and doubly ligated with heavy silk suture. The vessel then was cut between ligatures. The cut ends of artery retracted so that distance between ends was about 3 cm. In the same manner, the 2 common carotid arteries were doubly ligated and cut between ligatures. The cut ends of carotid vessels were about 6.5 cm apart. Of 7 dogs in the second group 2 had only the common carotid arteries cut, while 2 had both vertebrals severed. In the fifth dog, both common carotids and one vertebral artery were cut, and both vertebral and both internal carotid arteries were sectioned in the remaining 2 animals. Following operative procedure, the neck incision was closed in 2 layers, and the dogs returned to their cages. Heart rate was determined by auscultation on each anesthetized dog in Group 1 (carotids and vertebrals cut): a) prior to surgical intervention, b) immediately after neck incision was closed, c) one hour following operation, d) daily for one week, and then e) at irregular intervals thereafter. The eyegrounds of all dogs were examined by ophthalmoscope at beginning of experiment, while each vessel was being ligated, and subsequently at irregular intervals during post-operative period. Rectal temperature was determined previous to anesthesia, upon completion of surgery, and then once daily for a week following operation, after which monthly determinations were made. Behavioral changes were observed during initial weeks following operation; observations continue to the present (Aug. to Dec. 1959). The physical condition of the 2 groups of dogs was compared frequently. Except for one case, no special treatment was accorded any dog over that of others maintained in animal hospital. The one dog exhibited signs of acute distemper on second post-operative day, and 300,000 units of penicillin were administered twice daily for 2 days, after which the animal appeared to recover.

Results. Group 1. With one exception, heart rate of all dogs in this group decreased progressively as each vessel was sectioned. In this dog, there was a marked, though transient, increase in rate. One hour after incision was closed, the heart rate in each animal was below its pre-operative level. The results are summarized in Table I. All dogs exhibited a steady increase in heart rate with a return to initial normal levels approximately 3 weeks post-operatively. This interval probably reflects results of increasing collateral blood supply to the head, and correlates more or less with a return to more normal behavioral activity.

Daily intake of food and water was normal. No differences in fecal or urinary excretion compared with intact dogs were observed in the 2 groups of experimental animals.

Body temperature was slightly depressed below normal range of 100°-102.5°F following operation, but returned to normal limits about 24 hours in all but 2 dogs which maintained a consistent unexplained temperature between 103° and 103.6° F for 2 weeks following surgery. Temperatures eventually returned to normal. Daily temperature determinations during the post-operative week showed no significant departure from normal in the other 5 dogs.

The miosis produced by morphine made visualization of eyegrounds somewhat difficult. Nevertheless, no changes could be observed in caliber of retinal vessels as various arteries in the neck were tied, nor has any change become apparent in subsequent examinations. The color of optic disk, retina and tapetum remains normal throughout period of observation.

Tongue and mucous membranes of mouth became progressively more pale as each artery

TABLE I. Changes in Heart Rate Following Section of Carotid and Vertebral Arteries in 7 Dogs.

Pre-op. H.R.	H.R. after cutting both car. and vert. arteries	H.R. 1 hr post-op.	H.R. 24 hr post-op.	H.R. 2nd wk post-op.	H.R. 3rd wk post-op.
130	124	120	96	122	136
156	122	102	140	140	148
152	136	66	88	126	154
146	96	60	112	138	158
120	228	90	110	136	142
176	150	100	86	126	156
156	148	86	92	148	168

was tied. Following occlusion of last vessel, mouth and tongue were strikingly anemic and cool to the touch. There has since been an increase in color of these tissues, but they do not approximate their normal pink color when compared with that of intact dogs.

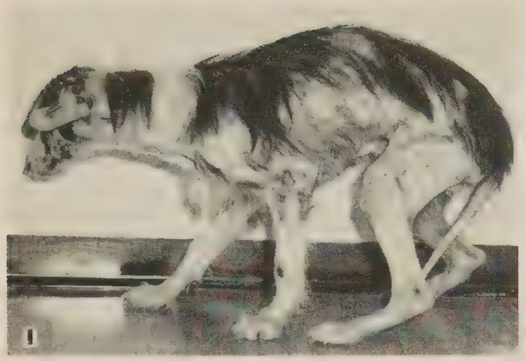
During 2 to 3 weeks following operative procedure, animals remained markedly lethargic and apathetic. Stimulation with pinpricks, pinching or loud noises failed to arouse them. They often were asleep with their heads buried, curled up in corner of cage. Although confined to individual cages in a room containing 60 other dogs that barked loudly as one entered the room, these dogs continued sleeping; or, if awake, would observe the investigator with no detectable sign of interest. When removed from cage for examination, they remained still.

From third week to the present, the dogs showed signs of increasingly normal activity, moving about, and evidencing normal curiosity when removed from cages. Pinching the footpad elicited normal withdrawal reaction. The dogs may wag their tails when approached, and show other overt signs of awareness and interest in their surroundings, but demonstrate little enthusiasm. Their gait is normal when led about on a leash. They do not display typical bravado of many long-caged dogs, *i.e.*, fierce barking, threatening attitude, etc. One of the animals selected for operation was regarded by animal caretakers as extremely vicious. Since the time of operation, this animal has become as docile as other members of the group.

The physical condition is striking after 4 months, in dogs of Group 1. They are markedly emaciated, with decrease in amount of hair over entire body, with a few denuded areas. Hair loss was first noticed beginning in the head, especially about the eyes, and gradually involved remainder of body. The remaining hair is harsh, dry and brittle, resembling that seen in hypothyroidism. The skin over most of body is white, dry and scaly. The ribs protrude giving a washboard appearance. Legs of all dogs are markedly edematous. The animals walk somewhat hesitantly. The skin over legs, unlike that of head and trunk, is moderately hyperemic, and covered with very little hair.

Group 2. The 7 dogs appear normal. Comparison of appearance of a dog from Group 1 and one from Group 2 may be observed in Fig. 1 and 2. The dogs in Group 2 are in good physical condition, normally alert and friendly, and the hair is full, bright and glossy.

Discussion. While most animals, including man, will not tolerate sudden occlusion of carotid and vertebral arteries, this procedure in the dog is not incompatible with life. A chronic experiment of this type was attempted by 2 other investigators(1,2). Cooper(1) ligated both common carotid and vertebral arteries in a dog which he sacrificed 9 months later. He observed none of the physical changes shown in our dogs subjected to the same operative procedure; he noted his animal "became a good house dog." It is well known that ligatures about blood vessels may become loosened or disappear with subsequent canalization of thrombi located at former site of ligature. It was to preclude this possibility that arteries of our dogs were sectioned rather than tied. Consideration of the excellent condition of all dogs in Group 2 lead to the assumption that canalization may have occurred in Cooper's dog with eventual restoration of



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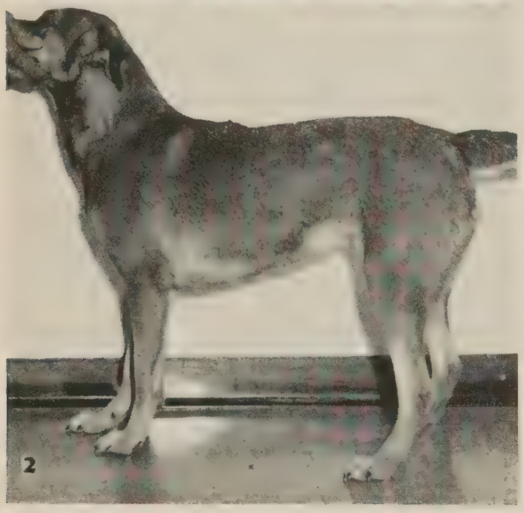

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FIG. 1. Dog from Group 1, 4 mo after section of both common carotid and both vertebral arteries.

FIG. 2. Dog from Group 2, 4 mo after section of both common carotids and one vertebral artery.

adequate blood supply to the brain. We showed that patency of even one of the 4 major arteries supplying the head does not result in changes observed after all 4 vessels are sectioned. Our findings are not in accord with the comment by Guyton(7) that the vertebral arteries alone cannot adequately supply the entire head with blood, although he notes later that it is difficult to determine the precise degree of cerebral ischemia after carotid and vertebral ligation.

It is evident from these observations that a rich collateral circulation to the head of the dog exists, and that it becomes functional very quickly to sustain at least some blood supply to vital tissues within brain stem and head. That the brain in such cases appears to be supplied preferentially before the remainder of the head might be expected because the initial remaining arterial supply is by way of the anterior spinal artery through the basilar artery to the Circle of Willis, and thence into internal carotid and through the anastomotic artery to the external carotid circulation. The anatomic basis for this observation is being reported separately.

Some confirmation of this selective pathway may be inferred from the following experimental observations. Blood vessels supplying the retina are direct branches of arteries arising from the Circle of Willis. After each of the carotid and vertebral arteries was sectioned, there appeared to be no observable change in diameter of these vessels. On the other hand, tongue and mucous membranes of mouth appeared strikingly pale following interruption of the 4 major arteries to the head. This subjective finding is strengthened somewhat by comparison of the macerated, latex-injected tongue of a normal dog with that of a dog whose carotid and vertebral arteries had been sectioned. While the tongue of intact dog showed normal, very profuse vascular supply to this structure, that of the operated dog was markedly deficient in number of injected vessels. Only about the sides and tip of the tongue did it resemble that of the normal control, the remainder showing many areas almost devoid of any but the largest branches of the lingual artery.

Hill(2) noted that retinal arteries appeared to be diminished in caliber, while veins were enlarged 3 days following ligation of carotid and vertebral arteries. His observation is difficult to interpret in view of our findings, and the fact that he did not section the arteries which he ligated. The possibility exists that the condition was present previous to ligation. He records no pre-operative observations.

The dull behavior of dogs in Group 1 might possibly be attributed to some disturbance in cortical function due to a decrease in blood supply to the brain. This aspect is being investigated, and histopathologic studies are being conducted to determine such structural changes as may occur.

No explanation based on experimental evidence is advanced at this time for edema of the extremities, emaciation or hair loss.

Summary. 1. In a group of 7 dogs, the common carotid and vertebral arteries were doubly ligated and sectioned. In a second group of 7 dogs, common carotids were cut in 2 animals, vertebral arteries in another 2, vertebrals and internal carotids in another 2, and common carotids and one vertebral in one dog. 2. Animals in both groups survived 4 months from August to December, 1959. Heart rates, body temperatures and eyegrounds remained essentially normal in animals of both groups. Of dogs in the first group (carotid and vertebral arteries cut), intelligence and alertness appear somewhat impaired, and physical condition is poor, with marked emaciation, hair loss, and edema of the extremities. All animals in second group appear normal in all respects. 3. The presence of known arterial anastomoses makes complete cerebral anemia most unlikely following section of both common carotid and both vertebral arteries in the dog. It is concluded that permanent interruption of the carotid and vertebral arterial supply to the head and brain of the dog is not incompatible with life, and that collateral circulation to the head is adequate to sustain life for at least 4 months.

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Effect of Insulin on Rate of Hepatic Uptake of NEFA.* (25604)

WILLIAM C. SHOEMAKER, JAMES ASHMORE, PETER J. CARRUTHERS AND MILTON SCHULMAN (Introduced by Heinrich Necheles)
Dept. of Experimental Surgery, Michael Reese Hospital, Chicago, Ill. and Dept. of Biological Chemistry, University of Indiana Medical School, Indianapolis, Ind.

Considerable attention has been given to the role of plasma albumin bound free fatty acids (NEFA/UFA) in mobilization and transport of lipids(1). The interrelation of plasma NEFA and carbohydrate has been emphasized(2,3). The observed fall in plasma NEFA in response to insulin has been attributed to an action of insulin to inhibit NEFA release from adipose tissue. This conclusion is based on the evidence: 1) that insulin inhibits release of NEFA from adipose tissue *in vitro*(4); 2) that insulin effects changes in plasma C^{14} NEFA specific activity(5); and 3) that glucose and insulin abolish A-V differences in plasma NEFA(6). Stein and Shapiro injected C^{14} palmitic and linoleic acid into the mesenteric vein of anesthetized rats (7). These studies have demonstrated that the liver rapidly removes labeled fatty acids from plasma and incorporates them into hepatic triglycerides and phospholipids. The present report describes the effect of insulin on the measured rate of removal of plasma NEFA by the liver in normal unanesthetized dogs.

Methods and materials. Eight experiments were performed on 4 male and 4 female mongrel dogs weighing 16 to 22 (average 19) kg. The hepatic vessels were catheterized with large plastic tubing by a previously described technic 2 to 7 days prior to experiments(8). All dogs were studied in an unanesthetized state without sedation after having been fasted overnight. Arterial, portal and hepatic venous blood was withdrawn by a constant aspiration pump, so that 4-6 contemporane-

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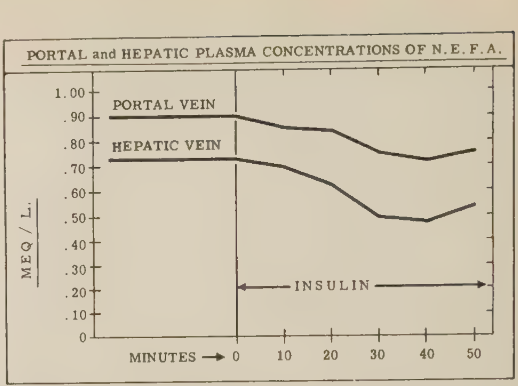
* Supported by research grants from USPHS and Eli Lilly and Co., Indianapolis, Ind.

ous, 10 minute integrated samples were obtained consecutively before and after insulin administration. In all instances blood from a donor dog was used to replace quantitatively the sampled blood. HGF-free insulin was constantly infused into the portal vein in doses of 0.0016 to 0.0033 u/kg/min. Hepatic plasma flow (HPF) was measured by a modified bromsulphalein method(9). NEFA determinations were made on 1 ml aliquots of the same plasma used for measuring plasma flow. The method of Dole was followed except that the Thymol blue indicator was titrated to the desired end point both before and after addition of the heptane extract. Thus, each sample had, in effect, its own blank. This was done in order to increase the accuracy of the concentration gradients measured on relatively small samples. Data presented here include: 1) measured concentrations of NEFA in arterial, portal and hepatic venous plasma; 2) portal-hepatic venous and arterial portal venous concentration gradients; 3) estimation of rate of hepatic uptake of NEFA obtained by multiplying the portal-hepatic venous plasma NEFA gradient by the HPF; 4) an approximation of output of NEFA from the splanchnic area. By making the assumption that ratio of portal venous blood flow to hepatic arterial blood flow is constant during the experiment, it is possible to obtain a first approximation of rate of splanchnic NEFA output. Blalock and Mason(10) found in the unanesthetized dog that portal vein blood flow comprised an average of 80.5% of total hepatic blood flow. NEFA output therefore was calculated by multiplying the arterial-portal venous plasma NEFA gradient by 80.5% of total hepatic plasma flow.

Results. Mean arterial plasma concentration of NEFA for the series was .84 meq/l during the control period. During the first hour after insulin administration, average arterial plasma NEFA concentration fell to .72 meq/l. The lowest arterial concentration for the series during this period averaged 0.55 meq/l. With one exception this nadir occurred at, or near, the end of the hour. Mean portal and hepatic venous plasma NEFA concentrations are illustrated in Fig. 1.



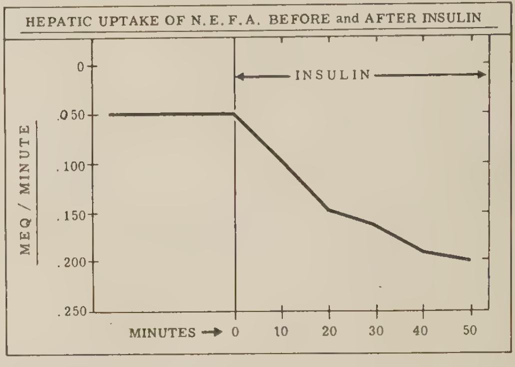


FIG. 1 (top). Mean values of portal and hepatic venous NEFA concentration before and after insulin administration.

FIG. 2 (bottom). Mean rate of hepatic uptake of NEFA during control period and as function of time after insulin administration.

Plasma concentration gradients of NEFA across the liver and across the non-hepatic splanchnic area are summarized in Table I. Mean hepatic portal-venous concentration gradient was negative in the control period (–.016 meq/l), indicating that the liver was taking up NEFA. This negative gradient increased to a mean of –.021 meq/l after insulin administration. Mean portal-arterial concentration gradient was obtained in 6 dogs of the series during the control period; in 3 of these dogs the gradient was positive; in 3, it was negative. In the control period mean portal-arterial gradient was .020 meq/l. After insulin this gradient decreased to a mean of –.002 meq/l.

When the values of all animals were taken together, there were sufficient observations both in the control period and after insulin administration for statistical evaluation. Expressed as mean ± S.E. calculated hepatic uptake of NEFA was 0.049 ± .016 meq/min

TABLE I. Mean Concentrations, Gradients and Output of NEFA before and after Insulin.

	Control	After insulin	Net change
NEFA portal vein	.90 meq/l	.77 meq/l	–.13 meq/l
" hepatic "	.74	.56	–.18
" arterial "	.84	.72	–.12
Hepatic-portal	– .16	–.22	–.06
Portal-arterial*	+.03	–.01	–.04
Hepatic NEFA output†	– .049 ±.016 meq/min.‡	–.150 ±.025 meq/min.‡	–.101 meq/min.‡
Splanchnic NEFA output†	+.020 "	–.002 "	–.022 "

* Based on 6 experiments only.

† Means and S.E. derived by considering all control observations of series together and comparing these with all observations made in experimental period.

‡ Uptake is represented as a negative output or gradient.

before and 0.150 ± .025 meq/min after insulin (P .001). Changes in hepatic NEFA uptake as a function of time after insulin are illustrated in Fig. 2.

The mean NEFA output of the non-hepatic splanchnic area was +.020 ± .016 meq/min before and –.002 ± .017 meq/min after insulin, an increment of +.022 meq/min over control levels. This change was not statistically significant.

Discussion. Studies with labeled fatty acids have indicated that these substances are rapidly removed from the blood and converted in part to triglyceride, presumably in the liver (7,11). The studies presented here have attempted to quantify rate of removal of NEFA by the liver in a control period before and after insulin administration. They indicate that after an infusion of glucagon-free insulin there is an immediate and pronounced increase in rate of hepatic removal of NEFA from portal blood. If it may be assumed that arterial NEFA concentration represents that of the extracellular phase and that the latter comprises 30% of total body weight, calculation of mean decrease of total extracellular NEFA is permitted. The calculated decrease in this series was found to be 0.68 meq. The mean net increase in hepatic NEFA uptake over that of the control period in this series was found to be 0.61 meq after insulin administration. Thus, the increase in rate of NEFA uptake by the liver was almost enough (90%) to account for the decrease in total plasma and extracellular NEFA observed in this series during the first hour after insulin administration.

In the data presented herein, the authors have been unable to find a statistically significant change in output of NEFA from the non-hepatic splanchnic area after insulin administration. These conclusions differ with those of other workers who have concluded from turnover studies and *in vitro* systems that the fall in plasma NEFA is due to a diminution of NEFA released from adipose tissue(4,5). The non-hepatic splanchnic area is comprised of intestinal tract, parenchymal organs as pancreas and spleen, and large stores of mesenteric and omental fat. The gradient, as well as net output or uptake of this area, therefore, represent the influence of both the gut and the intraperitoneal fat stores. The assumption has frequently been made that this splanchnic area is metabolically inactive in the fasting state. However, it has been demonstrated that this area is remarkably active in taking up glucose after insulin administration(12). We feel that at present time there are insufficient data to measure concomitantly the relative contribution of liver and body adipose tissue, *per se*, to the fall in plasma NEFA after insulin in the *in vivo* state.

An immediate effect of insulin which modifies hepatic lipid metabolism has been noted in a number of instances (13,14,15). If there is an action of insulin which increases hepatic fatty acid synthesis, such an effect would not necessarily be expected to increase NEFA uptake by the liver. If, however, fatty acid synthesis is stimulated by an increase in rate of esterification to triglyceride, as suggested by Balmain, *et al.*(16,17) for mammary tissue, then it is reasonable to expect that an increase in triglyceride formation would increase hepatic NEFA clearance. On the basis of these data one may be led to speculate or predict that there may be an increased hepatic output of triglycerides concomitant with the increased uptake of NEFA following administration of insulin.

Summary. Concentrations of NEFA in plasma of blood obtained from splenic artery, portal and hepatic veins were measured in unanesthetized dogs together with hepatic plasma flow. Rates of hepatic and non-hepatic splanchnic uptake or output of NEFA were calculated before and after insulin administration. A marked increase in rate of hepatic uptake of NEFA was observed after insulin administration. No significant change in rate of NEFA output from non-hepatic splanchnic area was found.

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Effects of Physico-Chemical State of Vit. B_{12} Preparation in Digestive Tract on Its Absorption.* (25605)

KUNIO OKUDA, ELBA DURAN V. AND BACON F. CHOW
Dept. Biochemistry, The Johns Hopkins University, School of Hygiene & Public Health, Baltimore, Md.

D-sorbitol in large doses can enhance intestinal absorption of Vit. B_{12} under appropriate conditions in man(1) and rats(2). This phenomenon may be (a) due to formation of a chemical derivative of sorbitol in the gastrointestinal tract which can enhance absorption (3), or (b) due to physical characteristics of the concentrated sorbitol solution. Therefore, some commercially available surfactants which are derivatives of sorbitol and the importance of the physical characteristics of the administered solution in enhancement of Vit. B_{12} absorption were studied. This communication reports the results of such studies and presents a hypothesis on the mechanism of enhancement of Vit. B_{12} absorption by such compounds.

Methods. Measurement of Vit. B_{12} absorption. Adult rats were fed by stomach tube 50 mμg or 1000 mμg of aqueous radioactive $Co^{60}B_{12}$ with a total radioactivity of 50 and 100 mμc, respectively. Absorption was measured by fecal recovery of radioactivity in 4 days, or only by organ uptake of radioactivity 2 days after administration when the latter dose was used. Determination of radioactivity in feces and organs has already been described(4). In some instances "Intestinal Loop" technic(5) was used.

Results. Effect of surface active agents on Vit. B_{12} absorption.† One group of rats was given by a stomach tube 1 cc of undiluted Tween 80 and immediately afterwards 1 cc of $Co^{60}B_{12}$ by another stomach tube. Another group of control animals received

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* Supported by Grant-in-Aid from U.S.P.H.S.

† Surface active agents used were obtained from Atlas Powder Co.

TABLE I. Effect of Tween 80 on Intestinal Absorption of $Co^{60}B_{12}$.

Study	B_{12} dose (mμg)	Group	No. of rats	Fecal absorption test (mμg)	Organ uptake of radioactivity (mμg)	
					Liver	Kidneys
A	50	Control*	3	17.6 ± 1.3	2.50 ± .06	1.95 ± .39
		Tween 80†	3	30.7 ± 2.7	4.92 ± .73	2.75 ± .39
B	50	Control*	5	14.0 ± 2.3	1.70 ± .37	1.49 ± .15
		Tween 80†	3	36.2 ± .3	3.75 ± .49	3.41 ± .29
C	1,000	Control*	3		8.8 ± .7	19.4 ±2.2
		Tween 80†	3		26.1 ±2.0	48.2 ±2.4
D	50	Control‡	4	22.1 ± .5	2.42 ± .40	6.30 ±1.52
		Tween 80‡ (diluted)	4	25.9 ± .2	2.43 ± .45	5.74 ±1.43

* One cc water immediately followed by 1 cc of $Co^{60}B_{12}$.

† One cc undiluted Tween 80 immediately followed by 1 cc of $Co^{60}B_{12}$.

‡ Ten cc water (control) or 1/10 diluted Tween 80 followed by 1 cc of $Co^{60}B_{12}$.

1 cc of water instead of Tween 80. The results (Table I) demonstrate clearly that administration of $Co^{60}B_{12}$ preceded by administration of undiluted Tween 80 results in greater absorption ($p<0.05$) at both doses of 50 and 1000 mμg. However, when the same amount of Tween 80 was diluted first 10-fold with water and administered, this effect was no more demonstrable, in spite of the fact that the same amounts of Tween 80 and $Co^{60}B_{12}$ were administered.

Several other surface active compounds with the nucleus of sorbitol (Span 85, Tween 20, Tween 85, G-1096 and G-672) as well as those without it (Arlacel A and Myrj 52) were tested. They were selected because of their hydrophile-lipophile balance values ranged widely from 1.8 to 16.9. The typical results from a series of experiments are presented in Table II. The results of Study A demonstrated that only G-1096 among the compounds tested increased absorption significantly, and Study B demonstrated ability of Tween 85 to enhance absorption. Taken collectively, the results show that not all sorbitol-containing compounds are capable of enhancing absorption, and confirmed our previous results that dilution of Tween 80 abolished the ability to enhance the absorption of Vit. B_{12}.

Since surfactants and $Co^{60}B_{12}$ were not premixed before feeding, it was of interest to examine the physical state of such a mixture, even *in vitro*. When any one of these 3 sur-

TABLE II. Effects of Various Surface Active Agents on Absorption of $Co^{60}B_{12}$.*

Study	Agents† (1 cc)	Chemical form	No. of rats	Fecal absorption test (mμg)	Organ uptake of radioactivity (mμg)	
					Liver	Kidneys
A	Control		3	21.7 ± 1.6	3.39 ±.14	1.71 ±.97
	Tween 80 (10%)	Polyoxyethylene sorbitan monooleate	3	23.8 ± 4.7	3.65 ±.65	1.93 ±.41
	Span 85	Sorbitan trioleate	3	22.2 ± .5	2.88 ±.15	1.62 ±.29
	G-1096	Polyoxyethylene sorbitan hexaoleate	3	36.2 ± 1.0	4.86 ±.29	2.62 ±.29
	G-672	Glycerol sorbitan laureate	3	23.6 ± 2.6	2.55 ±.83	1.43 ±.28
B	Control		5	17.4 ± 2.7	3.01 ±.14	2.17 ±.29
	Tween 80 (20%)		4	17.7 ± 1.5	3.11 ±.29	3.11 ±.26
	" 20	Sorbitan monolaureate	3	24.8 ± 2.2	3.02 ±.28	2.81 ±.58
	" 85	Sorbitan trioleate	3	37.8 ± .6	4.98 ±.09	5.51 ±.09
	Arlacel A	Mannide monooleate	3	31.0 ± 4.2	4.16 ±.81	3.86 ±.44
	Myrj 52	Polyoxyethylene stearate	3	25.2 ± 3.9	2.94 ±.33	4.86 ±.99

* 50 mμg $Co^{60}B_{12}$ administered orally.

† Undiluted surface active agents were used except Tween 80.

TABLE III. Effects of Salts on Absorption of $Co^{60}B_{12}$.

Study	Vit. B_{12} dose ($m\mu g$)	Group	No. of rats	Organ uptake of radioactivity ($m\mu g$) Liver	Kidneys
I	50	Control	3	1.54 ± .32	4.60 ± .72
		30% $MgSO_4 \cdot 7H_2O$—1 cc	4	2.14 ± .12	6.47 ±1.08
II	1,000	Control	4	8.0 ±2.4	12.5 ±2.8
		30% $MgSO_4 \cdot 7H_2O$—1 cc	4	17.7 ±2.0	31.5 ±4.5
III	"	Control	5	7.9 ±1.7	24.1 ±3.5
		4% $MgSO_4 \cdot 7H_2O$—1 cc	3	15.0 ±2.5	35.1 ±4.1
		25% $Na_2HPO_4 \cdot 12H_2O$—2 cc	4	17.4 ±1.3	37.8 ±2.1

factants which could enhance absorption was mixed with water in the proportion used, a very thick vaseline-like product was formed. The other surfactants which did not enhance absorption, either did not mix with water or increased fluidity without jelly formation. Thus, such a difference in physical characteristics may have some bearing on ability to enhance Vit. B_{12} absorption by reducing the rate of descent into the intestine.

Rate of passage of $Co^{60}B_{12}$ in digestive tract after administration. A study was designed to measure rate of passage of $Co^{60}B_{12}$ from the stomach in presence or absence of Tween 80. Twelve rats were divided into 2 groups: 6 received 1 cc of undiluted Tween 80 and then 1 cc of $Co^{60}B_{12}$. The other 6 controls received the same volume of water and $Co^{60}B_{12}$. Two rats in each group were sacrificed at 1, 2 and 4 hours after oral administration. The digestive tract was divided into stomach, proximal-, middle- and distal ⅓ of the small intestine, and cecum, and radioactivity in each section measured. It was found that 1 hour after administration, more than 70% of radioactivity was already in the distal ⅓ of the small intestine and only 1% remained in the stomach in control rats; in experimental group, the stomach still retained 53% of radioactivity. This trend of delayed passage of $Co^{60}B_{12}$ in the Tween 80 group was also apparent 2 and 4 hours after administration. The physical state of the mixture containing $Co^{60}B_{12}$ was such that the stomach could not evacuate it as fast as it could when present in solution.

Effect of salt solution on absorption. The effect of co-administration of Vit. B_{12} and a cathartic which may accelerate passage of Vit. B_{12} in the intestine was studied. When 1 cc of 30% $MgSO_4 \cdot 7H_2O$ was fed immediately before oral dose of $Co^{60}B_{12}$, absorption was enhanced (Table III). The effect was even more easily detectable when a larger Vit. B_{12} dose (1000 $m\mu g$) was used. When 2 cc of 25% $Na_2HPO_4 \cdot 12H_2O$ was used, absorption was also increased. However, when 1 cc of isotonic solution of $MgSO_4 \cdot 7H_2O$ (4%) was given, the effect was less marked. It was noted that the animals receiving hypertonic solutions of both salts developed mild to moderate diarrhea.

Effect of Tween 80 and magnesium sulfate on intestinal wall. We have recently described a technic using a surgically prepared intestinal loop for assessment of absorption of Vit. B_{12} in the presence of known amounts of intrinsic factor or other substances which influence absorption(5). This procedure was used to study the effect of surfactants and hypertonic salt solutions. In Study A, the effect of Tween 80 solution was compared with that of stomach extract when 50 $m\mu g$ of $Co^{60}B_{12}$ was administered. The results (Table IV)

TABLE IV. Absorption of Vit. B_{12} as Measured with "Loop" Technic.

Study	B_{12} dose ($m\mu g$)	Group	No. of rats	Radioactivity recovered ($m\mu g$) Loop	Liver	Kidneys
A	50	Control	4	47.1 ± .11	.96 ± .14	.72 ± .10
		Tween 80 (20%—0.5 cc)	6	49.2 ± .49	.64 ± .11	.24 ± .07
		Stomach extract	5	29.4 ± .88	5.40 ± .40	2.47 ± .46
B	1,000	Control	3	809 ±11	23.9 ±1.7	33.5 ±4.3
		$MgSO_4 \cdot 7H_2O$ (15%—1 cc)	4	941 ±10	6.0 ±1.0	5.2 ±2.1

indicate that absorption by the loop was not enhanced as it was when the whole animals were studied. There was even a slight decrease in absorption by Tween 80, as indicated by kidney uptake of radioactivity. One cc of 15% of $MgSO_4 \cdot 7H_2O$ markedly reduced absorption of the test dose of 1000 mμg (Study B).

Discussion. Absorption of Vit. B_{12} can be enhanced by a large dose of D-sorbitol, but the mechanism is not understood. It is possible that the active agent is some chemical derivative of sorbitol formed in the gastrointestinal tract. For this reason, we have studied the effect of some readily available derivatives of sorbitol with surface activity. It was found that pre-administration of certain but not all of sorbitol containing compounds would increase Vit. B_{12} absorption. This increase is not related to surfactant activity. The 3 tested surfactants with ability to enhance absorption (Tween 80, Tween 85 and G-1096) have the common physical characteristics of becoming a jelly-like mixture when mixed with an equal volume of water. Thus, the observed phenomenon may be due to the physical rather than chemical state. When the effective dose of Tween 80 was pre-diluted with water to a fluid state and then administered, no increase in absorption was observed. The aqueous $Co^{60}B_{12}$ solution will probably become a semi-solid mixture upon contact with one of the effective compounds in the stomach and the speed of descent of $Co^{60}B_{12}$ from the stomach is greatly reduced. Since no correlation was observed between chemical structure and enhancement of absorption, it is presumed that the time during which Vit. B_{12} remains in the stomach might play a dominant role in enhancement of absorption. It is conceivable that the slow passage in the gastrointestinal tract allows maximum time for intrinsic factor in the stomach to react with Vit. B_{12} or a longer reacting time between Vit. B_{12} and the intestinal mucosal receptor. The former mechanism seems unlikely because of the results of the experiment in which $Co^{60}B_{12}$ was allowed to be retained in the stomach for 2 hours by ligation of the pylorus and absorption of such a dose of Vit. B_{12} was not increased by fecal measurement or by organ uptake.

Intestinal absorption is a function of time; therefore, it is likely that absorption of Vit. B_{12} is dependent upon its mobility through the digestive tract. A slower descent of Vit. B_{12} in the intestine will provide a better chance for the receptor to bind with Vit. B_{12}. If this were the only controlling factor, administration of cathartics might have the opposite effect. Contrary to such expectation, absorption of Vit. B_{12} at 1000 mμg dose administered with a hypertonic salt solution resulted in absorption 2 times greater than that of Vit. B_{12} without the salt solution. Since both magnesium sulfate and disodium phosphate exerted a similar effect, the phenomenon may not be limited to these 2 salts. Uptake of $Co^{60}B_{12}$ by resting *Lactobacillus leichmannii* organisms(6) is inhibited by high salt concentrations *in vitro*. Addition of salt would favor intrinsic factor in its competition for Vit. B_{12} binding in the digestive tract with microorganisms. However, absorption of Vit. B_{12} in presence of magnesium sulfate from the small intestine when tested with the "loop" technic was markedly reduced. It is conceivable that absorption of 1000 mμg dose of Vit. B_{12} in presence of hypertonic salts is partly achieved from the cecal wall, and that hypertonicity of the salt administered orally is reduced by the time it reaches the active site of absorption and thus adverse effect of hypertonicity on the intestinal wall is minimized. D-sorbitol in higher concentrations also interferes with absorption of Vit. B_{12} from the small intestine by the "loop" technic(7). Therefore, the effect of hypertonic salt solution on Vit. B_{12} absorption is similar to that of D-sorbitol in that both exert absorption enhancing effect in its high concentrations when administered orally to intact rats and yet both inhibit absorption when applied directly into an isolated loop of the small intestine.

Summary. Two means of enhancing Vit. B_{12} absorption in rats have been described. It was found that certain surface active agents, such as Tween 80, Tween 85 and G-1096, which form a semi-solid matter with an equal volume of water markedly enhanced absorption of $Co^{60}B_{12}$ when fed by stomach tube immediately before oral dose of $Co^{60}B_{12}$. The effect was abolished upon dilution of the agent with water before administration. Oral Vit.

B_{12} preceded by one of these undiluted compounds evacuated from the stomach at a much slower rate than without pre-administration of the compound. Saline cathartics such as hypertonic solutions of magnesium sulfate and disodium phosphate also enhanced absorption of Vit. B_{12} when administered in the same way. However, when tested with "intestinal loop" technic, neither surfactants nor salt solutions were able to substitute for gastric intrinsic factor. The possible mechanisms for the observed phenomenon of enhancement of absorption have been discussed.

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Plasma Protein VII. Site of Degradation of Serum Albumin.* (25606)

F. B. ARMSTRONG,† S. MARGEN AND H. TARVER
Dept. of Biochemistry, School of Medicine, University of California, San Francisco

We reported(1) inability to find significant breakdown of homologous albumin labeled with C^{14} *in vitro* in any slice or homogenate system prepared from rat tissues, with the exception of spleen homogenate. These results are in accord with those obtained by Katz and Sellers(2), but disagree with those of Roberts and Kelley(3) who reported rapid breakdown of serum albumin in slices prepared from rat livers. However, it is necessary to postulate that breakdown occurs at some site in the animal to account for the rapid turnover of serum albumin observed in numerous investigations *in vivo*(4). It is possible that albumin and perhaps other plasma proteins continually 'leak' into the gastro-intestinal tract where they are hydrolyzed. Cellular barriers are permeable in many cases to plasma proteins. Thus, both albumins and globulins pass rapidly from plasma into lymph in all species which have been investigated(5,6,7); they pass from the circulation into the peritoneal cavity and back(8,9,10) and into milk (11,12). It has also been shown that, in the guinea pig, plasma proteins are transferred across the placenta to the fetus(13), and, as is well known from immunological investigations and recent isotopic work, globulins are absorbed from the gut of newly born animals (14). To demonstrate such a loss of albumin into the gut from the blood stream, 2 types of experimental method were used, (a) the washing method, and (b) the freezing method. In both methods rabbits were first injected with either albumin-I^{131} or iodide-I^{131}. In the washing method, the lumen of an isolated section of the gut was continuously washed for several hours with a dilute albumin solution; in the freezing method an isolated section of gut was injected with an albumin solution; the section was immediately excised and frozen in an acetone-dry ice bath. While still frozen the tissue was removed leaving the core of frozen albumin-containing solution. Distribution of radioactivity in the albumin solutions so obtained was investigated. It is assumed that any radioactive albumin passing from the circulating blood into the gut would be trapped in the large volume of unlabeled albumin solution, and in the short time before enzyme action was stopped with trichloro-

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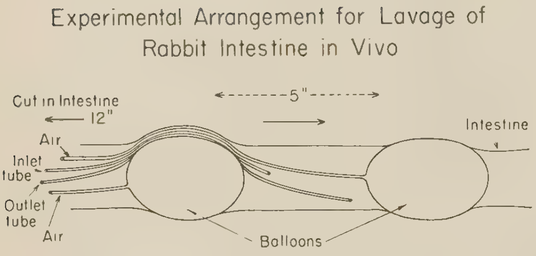
† Present address: Genetics Foundation, Dept. of Zoology, Univ. of Texas, Austin.

acetic acid (TCA), or freezing it would not be extensively broken down.

Methods. The iodinated albumin (bovine or rabbit, 100 mg) was prepared by a modification of the method of Gilmore and coworkers(17) and freed from excess iodide-iodine by dialysis against buffers containing a trace of thiosulfate and distilled water. Sufficient iodine was used to provide 4 atoms per mole of albumin. Assays for radioactivity were done in a well-type scintillation counter. The starved (24 hr) rabbits used in the washing-type experiments were sedated with morphine, then anesthetized with sodium pentobarbital. An incision was made in the abdomen, and a section of the intestine was removed and cut about 5 inches below the stomach. The apparatus (Fig. 1) was inserted in place about 12 inches below site of the cut. With this arrangement of balloons a section of the intestine was isolated, and the section was continually washed by circulating a solution through it *via* the small diameter polyvinyl tubing. Great care was taken not to damage the intestine during the operation so that no blood appeared in the section being washed. The washing solution used was 1% albumin (either bovine or rabbit) in Krebs-Ringer-bicarbonate buffer at pH 7.8, flowing at a rate of about 1 ml per min. Outflow was collected directly in an equal volume of 20% TCA. When the washing solution had been flowing for 30 min., the albumin-I^{131} or iodide was injected intravenously in amounts indicated in the captions to Fig. 2, 3, 4. Washing solution and blood samples were collected as indicated. Assays for radioactivity were made in the TCA-soluble and insoluble fractions. Small samples of the washing solution, obtained before precipitation with TCA, were tested for blood by the benzidine method. Experiments involving the freezing technic were carried out as follows: Each rabbit was lightly anesthetized and injected with albumin-I^{131} in amounts shown in Tables I and II. The animal was then left undisturbed for an hour, then deeply anesthetized. An incision was made in the abdomen, and a section of the duodenum was gently encircled at each end with 0.25 in. umbilical tape. The 2 pieces of tape were constricted, and albumin solution was injected carefully through a 27 gauge needle till the section was slightly distended. The section was then quickly excised and frozen in a dry ice-acetone bath. The intestinal wall was then peeled off following which the mucosa was scraped off, eventually leaving a frozen core representing contents of the intestinal lumen. After the core was thawed, the albumin solution was centrifuged to remove any remaining particles of mucosa, and solution was precipitated with TCA. Radioactivities were determined as previously noted. Electrophoresis of TCA-insoluble protein was carried out as follows: The precipitate was washed successively with 10% and 1%, TCA, then dissolved in 95% ethanol (18,19). Aliquots of the solution were subjected to zone electrophoresis on paper (Whatman #3 MM) in barbital-barbituric acid at pH 8.6, ionic strentgh 0.05, or pH 6.8, in sodium phosphate buffer 0.025 *M* for 18 hours at 200 volts. After drying, papers were stained with bromphenol blue (0.05% in 1% $HgCl_2$, 2% acetic acid). Then by making use of guide spots of serum albumin, the paper was cut into strips, which were counted in 4 ml of 0.05 *N* NaOH in a vial in the scintillation counter.

Results. Experiments involving washing of sections of the intestines of rabbits injected with either serum albumin-I^{131} or iodine-I^{131} are shown in Fig. 2, 3, 4. When labeled protein was injected a considerable amount of TCA-insoluble, as well as TCA-soluble material, appeared in the intestinal 'excretion' over the whole period of experiments (6.6 and 4 hr, Fig. 2, 3). On the other hand when labeled iodide was injected none appeared in TCA-insoluble form in the intestine (Fig. 4). The activity appearing in TCA-insoluble form could not be accounted for on the basis of blood in the lumen, because the benzidine test remained negative.

When the washing apparatus in the experiment on rabbit #10.0 (Fig. 2) was lowered to a previously unwashed section of intestine, in the following 30 minutes 2 x 10^5 cpm appeared in the washing solution. Of this activity 50% was TCA-insoluble. From this it must be concluded that appearance of activity in the intestine in the first 6.6 hours was



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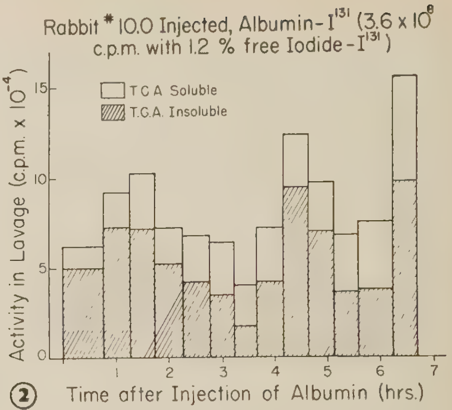

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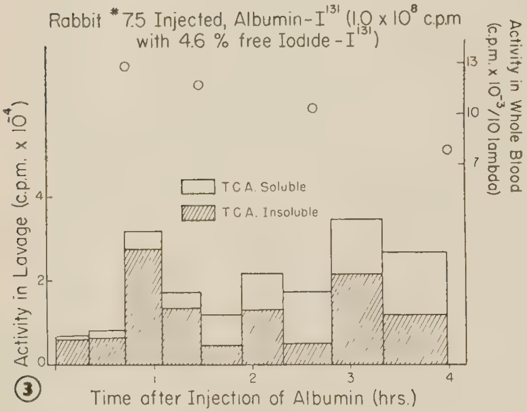

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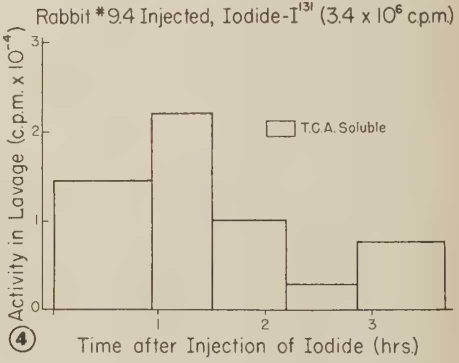

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FIG. 1. Experimental arrangement for washing lumen of an isolated section of intestine of rabbits.

FIG. 2. Distribution of radioactivity in washing fluid from lumen of an intestinal loop of rabbit 10 given serum albumin-I^{131} intrav.

FIG. 3. Distribution of radioactivity in washing fluid from lumen of an intestinal loop of rabbit 7.5 given serum albumin-I^{131} intrav.

FIG. 4. Distribution of radioactivity in washing fluid from lumen of an intestinal loop of rabbit 9.4 given iodide-I^{131} intrav.

not due to damage to the tissue.

From these experiments it is possible to make a rough estimate of actual amount of albumin appearing in the section of intestine. In the experiment with rabbit #10 mean specific activity of albumin in the blood over the 4 hour period of experiment was 5.4 x 10^4 cpm per mg, and mean 'excretion' rate was 10.2 x 10^4 cpm per hr for TCA-insoluble material. In terms of albumin this represents a minimum excretion of 2 mg per hour for the short section of intestine. In the case of rabbit #7.5 rate was 0.5 mg/hr.

If it is assumed that rabbit #10 (4.5 kg) had a total albumin of 13.5 g, with a half-life of 194 hours, then rate of breakdown was 48 mg/hr(20). In other words breakdown in the 13 cm section of intestine represented 4% of the total *in vivo* breakdown. In the rabbit #7.5 (3.4 kg) the observed rate of breakdown in the section was less but still comprised a significant fraction of the total. Therefore, from these experiments it must be concluded that, if excretion occurs elsewhere into the gut as comparable rates, a significant fraction of total albumin broken down by the rabbit may be accounted for by 'leakage' and breakdown in the gut.

The results of experiments carried out by the freezing technic are given in Tables I and II. In these experiments, as in the above, considerable radioactivity was found in the lumen of the intestine of rabbits (#6, 8, 4, 5) injected with albumin-I^{131}. Of this activity found in the lumen about 20% was TCA-insoluble. When this insoluble material

TABLE I. Transfer of Label from Albumin-I^{131} in Serum to Intestinal Lumen (Freezing Technic).

Rabbit	6	8	7*
Dose (cpm × 10^{-8})†	1.8	2.5	.03
Activ. in blood after 1 hr (cpm/2.5 lambda)	1600	604	None
Activ. in lumen (cpm), total × 10^{-4}	11.7	6.4	.06
TCA-insol. (%)	22	15	None

* Rabbit inj. with iodide-I^{131}.

† Free iodide in albumin-I^{131} preparations: Rabbits 6 and 8, 1.5 and 1.6%.

was subjected to electrophoresis, in the 2 cases investigated (rabbits 4 and 5, Table II), about 40% had the mobility and staining properties corresponding to those of serum albumin. About 20% moved from the origin but behaved differently from albumin, and the same per cent remained at origin, probably in the form of denatured protein. The remaining fraction, about 20%, was lost.

These results, although not amenable to quantitative interpretation, show that labeled protein with the properties of albumin appears in the lumen of the intestine of rabbits following injection of labeled albumin into the circulation. Since in these experiments the intestines were subjected to the very minimum of trauma, support is lent to the previous observations when the washing technic was employed. That part of the protein found in the lumen is actually the labeled albumin receives support from the properties of the protein. It is precipitable with TCA, yet TCA-protein is soluble in 95% alcohol, a rather unique property of albumin(18,19), and the protein moves with the same mobility as albumin at 2 pH values, 8.6 and 6.8 in different types of buffered solutions (Table II). When iodide, with radioactivity corresponding to that of the free iodide in the albumin-I^{131} given to rabbit 6, was injected into rabbit 7 no activity appeared in the TCA-insoluble fraction. Thus serum albumin does not acquire any firmly bound radioactivity when iodide-I^{131} is given to rabbits, confirming the results obtained in the washing type experiment with rabbit 9.4 (Fig. 4).

TABLE II. Nature of Label Transferred from Albumin-I^{131} in Serum to Intestinal Lumen (Freezing Technic).

Rabbit	4*	5
Dose (cpm × 10^{-8})‡	3.3	4.8
Length of section (in)	4	6
Total activity in section (cpm × 10^{-4})	14.0	8.9
TCA-insoluble (%)	23	22
Electrophoresis of TCA-insol. activity†		
Origin (%)	26	16
Albumin "	39	40
Remainder "	20	23

* Activity in blood at 1 hr 7,700 cpm/2.5 lambda.

† Zone electrophoresis on paper at pH 8.6. Similar results were obtained at pH 6.8.

‡ Free iodide in albumin-I^{131} preparation: Rabbits 4 and 5, 1.1 and 3.6%.

It should be mentioned that in patients with idiopathic (hypercatabolic) hypoproteinemia there is a great increase in permeability of the gastro-intestinal tract, so that these patients lose the polymer, polyvinyl pyrrolidone (PVP) into the feces when this substance is injected into the blood(15,16), as shown with the iodinated polymer. Subjects with other types of disorders not involving the gastro-intestinal tract were found to lose very little PVP from the circulation into the feces. Although PVP has an average molecular weight of 40,000, it appears possible that the substance does not behave like albumin, and thus might not be lost into the gastro-intestinal tract in normal subjects.

Summary. 1. Experiments have been carried out to show that serum albumin labeled with I^{131} passes from the circulation of rabbits into the duodenum. 2. Following injection of albumin-I^{131} the presence of TCA-insoluble activity in a solution passed through the lumen of isolated section of intestine can be shown. 3. In other experiments of a somewhat different type, the same transfer has been shown. 4. Material transported appears to be albumin since it is precipitated with TCA, the precipitate is soluble in 95% alcohol (like similar precipitate of pure serum albumin), and the soluble material possesses the same electrophoretic mobility as albumin in experiments carried out on paper. 5. Approximate calculations indicate that if this type of transfer occurs in a considerable part of the gastro-intestinal tract, then the enzymatic breakdown of serum albumin so transferred could account for most of the serum albumin breakdown which occurs *in vivo*.

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Etiologic Studies of Trachoma on Taiwan.* (25607)

J. THOMAS GRAYSTON, SAN-PIN WANG AND PAUL B. JOHNSTON
(Introduced by Robert A. Phillips)
U. S. Naval Medical Research Unit No. 2, Taipei, Taiwan (Formosa)† and Dept. of Medicine, University of Chicago School of Medicine, Chicago, Ill.

The etiologic agent of trachoma has for many years been considered to be a virus of the psittacosis-lymphogranuloma venereum (psitt.-LGV) group. Until recently strains of elementary bodies probably representing viruses of this group which were isolated in the yolk sac of eggs from trachoma patients were lost or not confirmed(1). Recent improvements in technic, particularly incorporation of streptomycin, have resulted in isolation of viruses thought to be the cause of trachoma (2), which have been confirmed(3). The adenovirus group have been shown to be an important cause of eye infections(4) and they are known to be frequent causes of acute conjunctivitis in Taiwan(5,6). Adenoviruses have been isolated from cases of trachoma in Saudi Arabia and even considered as a possible cause of the trachoma syndrome(7,8). It is also possible that adenoviruses may complicate or cause part of the clinical picture or contribute to the spread of trachoma through seasonal acute conjunctivitis. Utilizing the newly perfected technics for yolk sac isolation of elementary body agents and standard tissue culture technics for adenovirus, a study was undertaken of the viral etiology of trachoma on Taiwan.

Materials and methods. During October and November of 1958, specimens were collected from first grade school children in 15 of the 16 counties of Taiwan, including the Pescadore Islands. A total of 1600 eye swabs, throat swabs and blood specimens were collected from children on whom a diagnosis of the condition of their eyes was made by oph-

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† Mailing address: NAMRU-2, APO 63, San Francisco, Calif.

thalmologists attached to the Government of the Republic of China-World Health Organization trachoma control project on Taiwan (9). For the purpose of this control project, 3 categories of diagnosis were used, trachoma, conjunctivitis and nothing pertinent (N.P.). The trachoma diagnosis followed the modified MacCallan classification as recommended by the WHO expert committee on trachoma(10): I = trachoma at onset, II = established trachoma, III = cicatrizing trachoma and IV = cicatrized or healed trachoma. In addition, trachoma d was used for suspected but not definite trachoma. Almost all those children with the diagnosis of conjunctivitis had chronic follicular or papillary conjunctivitis which was thought to be non-trachomatous usually on the basis of lack of corneal lesions visible with a monocular lens. A few conjunctivitis cases appeared to be acute. The N.P. diagnosis included normal appearing eyes and pathology not due to infections. All children, except those with the diagnosis of N.P. or trachoma IV, were treated with antibiotic eye ointment under the control project. The eye swab and throat swab cultures were taken with dry cotton tipped applicator sticks, then immersed in individual screw capped vials containing 2.5 ml of brain heart infusion broth pH 7.3, without antibiotics for eye specimens, and containing 1000 units of penicillin and 1 mg of streptomycin per ml for throat specimens. The specimens were frozen in dry ice until returned to the laboratory, where refrigeration was continued at –65°C in a mechanical freezer. *Adenovirus isolations*: The technic for isolation and identification of adenoviruses used in this laboratory has been reported(11). HeLa cell tissue cultures were employed and each specimen observed for a minimum period of 30 days prior to being declared negative. *Trachoma virus isolation*: Eye specimens were unfrozen in a 37°C water bath and streptomycin added. The specimen was then allowed to stand at 4°C for 4 hours after which 0.2 ml containing 4 mg of streptomycin was inoculated into the yolk sac of each of 5 embryonated eggs, 6 to 8 days of age. The eggs were incubated at 35°C and candled daily. Upon death, or survival for 12-13 days, the yolk sac of the egg was harvested and an impression smear was stained by a modified Macchiavello method(12) for demonstration of the presence of elementary bodies. Each egg was cultured on blood agar for bacterial contamination. Two yolk sac subcultures were made of negative material. Eggs positive for elementary bodies were passed separately. Positive isolations were associated on passage with increasing numbers of elementary bodies and with death of embryos.

Results. Adenovirus isolations: A total of 922 eye specimens and 878 throat specimens from 6-7 year old children representing all areas of Taiwan were cultured for presence of adenovirus. One adenovirus was isolated from the eye specimens of 468 children with trachoma, 4 were isolated from 374 in the conjunctivitis category and none were isolated from 80 N.P. children, for an isolation rate of 0.5% from the 922 eye specimens. A total of 27 adenoviruses were isolated from the 878 throat specimens examined, for an isolation rate of 3.1%. The isolations were apparently unrelated to the diagnosis of eye condition, since the percent of specimens positive for adenovirus by diagnosis was 4.0 for trachoma, 1.8 for conjunctivitis and 4.6 for N.P. The adenovirus types isolated from eyes included 2 type 8, 2 type 4 and one type 3. A wide variety of adenovirus types was isolated from the throat specimens, including all types from 1 to 10, except types 4 and 9. The most frequently found types were type 10 isolated from 10 specimens and type 5 isolated from 5. One child with type 8 isolated from the eye specimen also had type 8 in his throat specimen. The throat specimen of one child with type 4 in the eye yielded type 5 adenovirus.

Trachoma virus isolations. A total of 4 elementary body virus strains was isolated from 100 eye specimens cultured through 3 passes in the yolk sac of embryonated eggs. A total of 121 specimens was cultured, but 21 were lost by contamination. Isolation methods detailed above are now used, but some of the earlier specimens in this study were cultured without being frozen and with as much as 8-fold less streptomycin. These earlier specimens were more frequently contami-

TABLE I. Trachoma Virus Isolations from Eyes of First Grade School Children on Taiwan in Relation to Diagnosis of Eye Disease.

Clinical eye diagnosis*	No. examined	Positive
Trachoma d	17	0
I	11	0
II	32	4
III	15	0
Conjunctivitis	20	0
N.P.	5	0
Total	100	4

* Modified MacCallan classification of trachoma except d = suspected or questionable trachoma.

N.P. = nothing pertinent.

nated. Table I shows that the 4 isolations came from among a group of 32 children with clinical diagnosis of stage II trachoma. There were no isolations from 43 children with other stages of trachoma, 20 with conjunctivitis or 5 with N.P. It is of further interest that 3 of the 4 isolations came from among 10 patients especially selected by the ophthalmologists as having particularly active disease. Elementary bodies were seen on first passage of 2 of the strains isolated and on second passage of the other 2. All strains killed embryos on second passage. Two strains have been through 19 and 16 passes respectively without any gross changes except some increase in egg pathogenicity. On yolk sac smear elementary bodies resembling those of the psitt.-LGV group are regularly seen.

The 4 Taiwan elementary body strains were shown to be related serologically to the psitt.-LGV group by complement fixation test. Table II presents the results of testing the 4 trachoma strains as antigen with a LGV, a psittacosis and a trachoma antiserum. The LGV serum was from a patient with the disease, the psittacosis serum was from an immunized guinea pig and the trachoma serum from an immunized rabbit. The 4 viruses fixed complement with each of the 3 serums. With the LGV and psittacosis serums, the trachoma strains produced a lower antibody titer than the homologous antigens.

Discussion. Isolation of adenovirus from the eyes of only 0.2% of children with trachoma and 1.1% with conjunctivitis, mostly chronic, would suggest that adenoviruses do not cause either syndrome on Taiwan. Concurrent isolation of 3% adenoviruses from throat specimens of the same children would indicate that the low isolation rate from eyes was not due to inadequate technics of specimen collection, storage, or culture. The adenovirus types isolated from the eye specimens, types 3, 4 and 8 have been frequently isolated from acute conjunctivitis in Taiwan (5,6), while type 3 and 8 have been associated with acute eye disease in other parts of the world(4). The adenovirus types isolated from the throat specimens have been less consistently associated with disease and the significance of these isolations will be considered in a separate report, in conjunction with studies of isolations of adenoviruses from various clinical syndromes on Taiwan. The frequency of adenovirus isolation from eyes of 6-7 year old children on Taiwan agrees closely with that reported from Saudi Arabia(7) for children over 3 years of age. If adenovirus eye infections are usually acute and the viruses present for only several days, a prevalence of 0.5% at one time could represent a considerable incidence during a year.

It is noteworthy that isolation of elementary body virus strains in the yolk sac of eggs

TABLE II. Serological Relation of Taiwan Trachoma Viruses (TW) to the Psittacosis-lymphogranuloma Venereum Group in Complement Fixation Test.

	Serum antibody titers						
	Antigens						
Antiserum	TW-10	TW-21	TW-89	TW-97	LGV	Psitt.	NYS
LGV patient	16	16	8	16	128		4
Psitt. guinea pig	32	64	32	64		256	4
TW-10 rabbit	256	256	256	256			8

Titers expressed as reciprocal of original serum dilution, +++ end point, NYS = normal yolk sac. Trachoma antigens are phenolized, heated 2½% yolk sac suspensions. LGV and psitt. antigens are commercial preparations (Markham). Psittacosis guinea pig serum prepared by immunization with the Markham antigen, human pneumonitis strain. Rabbit immunized with 20% yolk sac suspension of TW-10.

from patients with trachoma is associated with the most active cases. The failure to isolate these viruses from normal appearing eyes, from children diagnosed as having non-trachomatous conjunctivitis and from less active cases of trachoma adds one type of evidence to support the contention that these viruses are the etiologic agent of trachoma. This finding suggests that the clinical diagnostic problems of trachoma, which are usually the less typical and less active cases, would not be helped by virus isolation attempts at this stage of development of isolation technics. Collier (13) has reported isolation of 14 strains of elementary bodies from 14 persons with inclusion body positive trachoma. Snyder *et al.* (14) have isolated 14 trachoma strains, all from persons whose conjunctival cells showed inclusions. In this laboratory trachoma virus has been reisolated 22 times out of 23 attempts beginning 2 weeks after infection, from 6 human volunteers who have been infected with one of the Taiwan trachoma strains in a study to be reported. In these volunteers typical inclusions have been present on conjunctival cell smears at all periods when virus has been isolated. Conjunctival cell smears were not obtained on the first grade school children in this study. However, studies subsequently made in similar groups suggest that the proportion of trachoma that is inclusion body positive in first grade school children in most areas of Taiwan is only about 1%. This is probably the reason for the small number of isolations in this study. One additional Taiwan strain (TW29) has been isolated from an adult male with an active gelatinous stage III trachoma which was inclusion body positive.

In addition to the viruses from China(2) and Africa(3), similar elementary body strains of the psitt.-LGV group have been isolated from trachoma patients in Saudi Arabia (14) and Israel(15). On the basis of morphology and serology the Taiwan strains belong to the psitt.-LGV group and they are probably similar to those being isolated in egg yolk sac in other parts of the world. We believe the Taiwan elementary body strains are trachoma viruses because of the controlled type of isolation studies used and because the disease has been reproduced in 6 human volunteers with one of these strains.

Summary. Eye specimens from first grade school children on Taiwan were cultured in yolk sac of embryonated chicken eggs and in HeLa cell tissue cultures. Four viruses, morphologically and serologically related to psittacosis-lymphogranuloma venereum group, were isolated in yolk sac from 32 children with stage II trachoma, while negative results were obtained with similar cultures of 68 specimens from other stages of trachoma, conjunctivitis and uninfected eyes. These viruses are thought to be etiologic agents of trachoma. One adenovirus was isolated from 468 children with trachoma and 4 adenoviruses were isolated from 375 children with chronic conjunctivitis. Within limitation of age group studied, it is concluded that adenoviruses do not play an important role in trachoma syndrome on Taiwan.

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Visually Evoked Slow Negativity in Rabbit Cortex.* (25608)

Alan L. Pearlman,† Sidney Goldring and James L. O'Leary
Division of Neurosurgery and Beaumont-May Inst. of Neurology, Washington University School of Medicine, St. Louis, Mo.

A negative spike of 15 msec. duration can be evoked by direct activation of cerebral cortex, and a corresponding one (called recruiting response) by serial stimulation of the midline thalamus. In recent work(1,2,3) with D.C. recording we observed a slow negative wave which follows in the wake of each of these primary responses as a separate excitatory process of higher threshold, longer duration (250 msec.), marked propensity for summation upon repetitive stimulation, and longer than usual persistence following the end of a brief series of stimuli. The evidence for separate identity between spike and secondary process made it important to decide if such a late potential could also be evoked by stimulation of one of the paths leading from sensory end organs to cerebral cortex. We selected the evoked visual response as a test case, eliciting it both by optic nerve and light flash stimulation. The slow negativity alluded to had already been observed to follow the primary visual response. Bishop and O'Leary(4) observed it in rabbit cortex using a capacity coupled amplifier, and Goldring and O'Leary(5) saw it in D.C. records obtained by chopping an ink writer trace mechanically. However, in these prior studies its characteristics did not seem sufficiently unique to focus further attention upon it. In the present experiments upon the rabbit the slow negativity is a prominent potential, and accumulating data indicate its similarity to the slow processes previously shown to follow the direct cortical response and the recruiting wave(2,3). Hereafter we describe it and relate it to other electrical events of visual cortical response.

Method. Thirty-two rabbits weighing 1.5 to 2.0 kg were prepared under ether anesthesia after which all wound surfaces were thoroughly infiltrated with 1% Procaine HCl. The animals were then immobilized with Flaxedil and respired artificially. Recording began one hour following discontinuance of ether. In one group of animals a needle insulated to near the tip was inserted into the optic nerve and another having 7 mm tip exposure was placed in the periosteum; nerve stimulus strengths varied between 0.8 and 3.0 V., pulse duration being 0.1 msec. (square wave through isolation transformer). In the other group the retina was activated directly by unfiltered 10 μsec. flashes from a Grass photic stimulator. An artifact for signalling the flash was obtained by leading from the output of a photoelectric cell to jack positions adjacent to those for the recording electrodes. Transcortical records were obtained from visual cortex using non-polarizable calomel electrodes and a D.C. amplifier, oscillograph and camera.

Results. Between threshold and maximal response to direct nerve stimulation the span was 0.9 to 3.0 V. When stimulus strength was raised by steps from a sub-threshold level the first sign of activation noted was a 250 msec. slow negative potential—*slow negativity* (Fig. 1A, 4). At somewhat above threshold for the slow negativity an earlier small positive deflection appeared, signalling initiation of the primary visual response (Fig. 1B, 1). That positive could also be seen with lower stimulus values but was then impossible to distinguish as a specific response component from similar pips which appeared spontaneously in the record of cortical activity (Fig. 1A, 1?). With further increase in stimulus strength the positive phase of primary response (component 1) and the slow negativity (component 4) were noted to grow together in amplitude (Fig. 1B and C), then the negative phase (component 2) of primary response and its ensuing after-positivity (component 3) also developed as stimulus strength increased, (Fig. 1D). Past Fig. 1C (1.3 V.) no further

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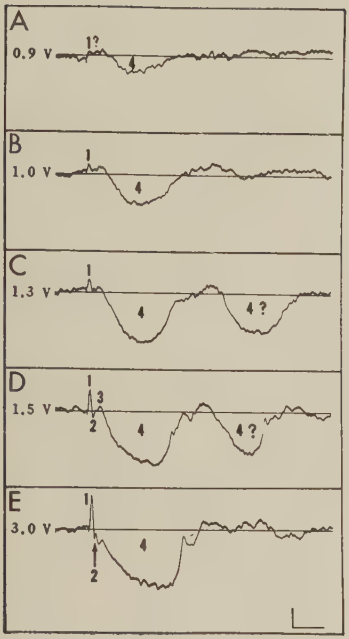
† Student trainee, Nat. Inst. Health.



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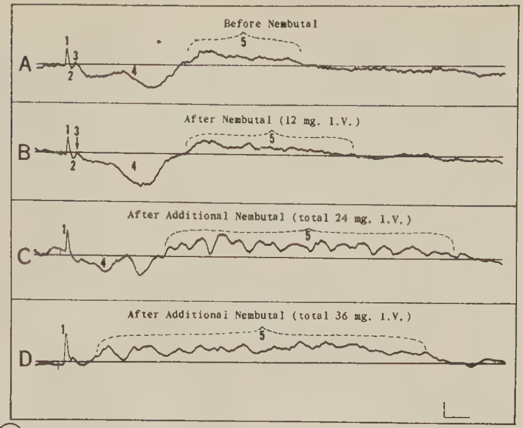

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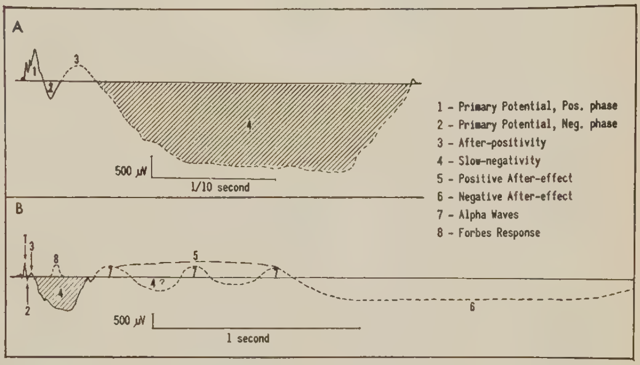

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FIG. 1. Visual cortical responses to optic nerve stimuli of increasing strength. Numbers to left of each row refer to stimulus voltage. 1, positive phase of primary evoked potential. 1?, suspected of being positive phase of primary response. 2, negative phase. 3, after-positivity. 4, slow-negativity. 4?, may represent recurring slow negativity. Straight horizontal line in this and subsequent figures is arbitrary baseline against which potential changes are read. Up is positive, down is negative. Cal. key in lower right hand corner: vertical line of right angle = 500 µV; horizontal line = 100 msec. Shock artifacts retouched; otherwise photographs are those of original record.

FIG. 2. Effect of deepening Nembutal anesthesia on optic response evoked by flash stimulus to retina. I.V. = intravenous. 1, positive phase of primary evoked potential. 2, negative phase. 3, after-positivity. 4, slow-negativity. 5, positive after-effect. Cal. key in lower right hand corner: Vertical line of right angle = 500 µV; horizontal line = 100 msec. Shock artifacts retouched; otherwise photographs are those of original record.

FIG. 3. *A*. Evoked primary visual response and slow-negativity diagrammatically represented on time scale sufficiently fast to permit detection of positive spikes on ascending limb of primary positive potential. *B*. Diagrammatic representation of all cortical potentials known to occur with an optic nerve stimulus. Latencies, amplitudes and duration of these potentials and their temporal relationship to each other are those which obtain in actual experimental situation. Figure constructed by schematically adding potentials 4?, 5, 6, 7 and 8 to composite potentials 1, 2, 3 and 4 which is actual record taken from Fig. 1, D.

augmentation of the slow negativity (component 4) occurred even though stimulus strength was raised to 3V., which was maximal for the initial positive phase (component 1). This evidence suggests that whatever excites the slow negativity has a somewhat lower threshold than that of the majority of elements responsible for the primary phase of the response.

The latency of slow negativity varied from one animal to the next but approximated 50 msec. The main factor which contributed to variability appeared to be the after-positivity (component 3) following the negative phase of the primary response (component 2). If sufficiently well-developed, that after-positivity can cut into the rise of the slow negativity thus lengthening the apparent latency of the latter. In addition, latencies appeared longer when the stimulus was near threshold. However, for any one experiment the latency for slow negativity remained constant for stimuli between half maximal and maximal strength (compare Fig. 1C, D and E).

Occasionally the response to a single nerve stimulus, submaximal for the primary visual response, showed a replica of the slow negativity component following the latter at a short interval (Fig. 1C and D, 4?). However, with maximal stimuli the apparent repetition of the slow negativity usually did not occur. The relation such repetition bears to the rhythmic alpha waves(4,8) which may succeed the evoked response is under study.

A slow negativity component also followed the primary visual response to photic stimulation (Fig. 2A, 4), the duration varying between 250 and 400 msec. That slow negativity, which was evoked by light flash, was followed by an equally enduring slow positive potential (Fig. 2A, 5) and thus differed from the response evoked by a shock to the nerve. We believe this slow positive potential to be identical with the positive after-effect of visual response described previously(6).

Activated by either nerve or flash stimulation the slow negativity was the most susceptible of the components to progressively deepening Nembutal anesthesia. Increments of 12 to 20 mg by successive intravenous injections to a total of 30 to 60 mg caused the slow negativity to disappear gradually. With disappearance it came to be replaced by the long positive after-effect of the primary response. Concurrently the primary response itself might increase somewhat in amplitude (Fig. 2, B through D).

Experiments to define the characteristics of the slow negativity are in progress. Thus we have shown slow negativity to summate in response to low frequency light flashes but (so far) not to a succession of nerve stimuli, to originate beneath the superficial layer of cortex, and to grow in size with surface applications of gamma amino butyric acid. These also characterize the slow negativities which occur as components of the direct cortical and recruiting responses(2,3).

Discussion. Recorded under optimum conditions slow negativity appears to be a unique feature of evoked response. It is actually a more prominent part of the evoked trace than is the primary component. This is graphically illustrated in Fig. 3A, primary visual potential and ensuing negativity being reproduced schematically upon a scale sufficiently rapid to permit analysis of the faster components of primary potential. It is important that all parts of the visual response do not become evident in any one preparation because of differential susceptibility of the components to anesthetic level; some appear with very light anesthesia, others reveal themselves only during deeper stages. However, a composite (Fig. 3B) can be constructed to reveal the temporal and amplitude relations between slow negativity and other components of visual response. These are identified as follows, with the significant features of each: *1,* positive phase of primary response assigned previously to activity in cell bodies and basal dendrites of deeper cortical layers(7,8); *2,* negative phase of primary response, presumed to signal antidromic activation of apical dendrites; it has a greater sensitivity to anesthesia than does the positive phase(7,8); *3,* after-positivity following the negative phase, a poorly understood part of the potential-complex having variable size and duration; *4,* slow negativity, seen only under light anesthesia; *5,* positive after-effect, seen best under moderately deep barbiturate anesthesia, during hypoxia or after repetitious episodes of convulsive activity(5); *6,* long-lasting negative after-effect usually associated with cortical activation(5); *7,* recurrent alpha waves(4) or so-called physiological after-discharge(8); *8,* Forbes "secondary" response, seen best under deep barbiturate anesthesia; it may also be recorded in the unanesthetized animal(9).

Summary. Visual cortex responses to optic nerve and retinal stimulation were studied by D.C. recording upon slow time line. A prominent slow negative wave (250 to 400 msec. duration), slow negativity, occurred in the wake of primary visual response. Evidence

indicates that when activated by nerve stimulation it has a threshold somewhat lower than that of primary response. Under moderately deep Nembutal anesthesia, slow negativity (evoked by optic nerve or light flash stimuli) disappears and is replaced by a longer lasting positive after-effect. The slow negativity described under these conditions is believed to have an origin in the cortex identical with corresponding slow negative waves evoked by direct surface and midline thalamic stimulation. In Fig. 3 it is placed schematically with relation to other components of visual cortical response previously described elsewhere.

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New Type Concentric Stimulating and Recording Electrode for Electroencephalography and Oscillography. (25609)

H. J. DAIGLE, L. C. MASSOPUST, JR. AND E. C. HANNAN*
(Introduced by H. S. Mayerson)
Dept. of Physiological Sciences, Southeast Louisiana Hospital, Mandeville, La.

Multipolar electrodes for chronic or acute implanting within the nervous system of experimental animals have always presented problems to investigators of bio-electric phenomena. Some of these problems are: 1) Multipolar metal, glass or twisted wire concentric electrodes are difficult to fabricate because of the problems of insulating one pole from another. Secondly, breakage, shorting and high impedance are common in all of the above types of electrodes. 2) Further, in constructing the concentric electrode of "non-micro" types of more than 2 poles, the diameter of the whole electrode increases in size in such proportions as to be impossible to use in animals with relatively small brains. To overcome some of the above difficulties, a new type of multipolar concentric electrode was developed which employed coats of silver printed circuit paint alternating over coats of insulating material.

Materials. The following materials are necessary for construction of the silver paint type concentric electrode. 1) Straight surgical or hypodermic needle stock of any desired diameter which will give proper rigidity for insertion into nervous tissue by means of stereotaxic devices. 2) Pure silver printed circuit paint. This can be obtained from any electronic parts dealer. 3) Silver solder (No. 430) of the type made by the All-State Welding and Alloys Co., White Plains, N. Y. 4) All-State flux (non-acid) for ferrous and non-ferrous alloys. 5) Insulating baking varnish which resists acids, alkalies, solvents and degreasers. The use of "Epoxylite" made by the Epoxylite Corp., El Monte, Calif. was found to be the best. 6) Lead wire of the miniature variety such as "Tensolite Miniature Wire" manufactured by the Tensolite Insulated Wire Co., Tarrytown, N. Y.

Methods. 1) Arbitrary lengths of steel needle stock were cut to any practical length. Actual lengths were determined later by the depth of structures in the brain stem to be stimulated or recorded from (See Step 4 below). Length and diameter used by the authors for insertion into the brain stems of cats were 2¾ inches and .025 inch respectively. Silver solder was applied by dipping the electrode first into flux then into melted solder and slowly withdrawing it. When cool, the tips of the electrodes were examined to make sure a homogeneous coating covered them. The tips were then cleaned and degreased, by use of such solvents as xylol and 95% ethyl alcohol. 2) The base end of the electrode was now inserted into a special cork holder for mounting in a mechanical device for applying thin, even coats of insulation over the steel core. This device consisted of a slow, constant-speed (1.16 rpm) electric motor, a rack and pinion and the electrode holder. The electrode core was lowered into a test tube containing Epoxylite, the motor turned on, which withdrew the electrode at a constant speed (1 cm/15 sec.) from the test tube. This operation was repeated 6 times, coating the steel core with micro-thin layers of Epoxylite. A few minutes were allowed to pass between Epoxylite coats to allow each coat time to set. 3) The electrode was then mounted in a vertical position and placed in a drying oven set at 50° to 60°C for a minimum of 24 hours. The longer the electrode is allowed to stay in the oven the harder the Epoxylite coating became. 4) After the first Epoxylite coatings were thoroughly hardened, the electrode was cut to actual length. These actual lengths of electrodes can be determined directly from any acceptable atlas of the brain of the experimental animal being used. After adding the thickness of the skull to the determined length, a point was found where the first lead off wire from the deepest pole was to be soldered to the electrode, each additional lead wire juncture point being distal to this point. One millimeter separations were found to be satisfactory for all additional poles. For example, in cats, length of a bipolar electrode necessary to reach the anterior or posterior hypothalamic area was 22 mm. To this was added another 1.5 mm to allow for the thickness of the skull and 2 more mm to keep the lead off wire joints above the surface of skull. 5) The first coat of pure silver paint was applied over the first insulation coat of Epoxylite with an artist's fine brush and with the aid of a relatively high power binocular dissection microscope. When dry, the silver paint layer was examined carefully to insure complete coverage of the underneath insulating layer. If there were gaps in the silver coat, the entire process was repeated. This silver coat was allowed to dry for at least 2 hours, then the second lead off wire was attached by lightly winding the wire around the silver paint layer near the "chuck" end of the coating. Next, a generous amount of silver paint was applied over the winding and continued down into the silver coating itself. This was allowed to dry for at least one hour. 6) The recording or stimulating tip of the steel core was now scraped clean of the covering layer of Epoxylite to any predetermined exposure and dipped into a thick grease such

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* Present address: 8th Naval Dist., New Orleans, La.

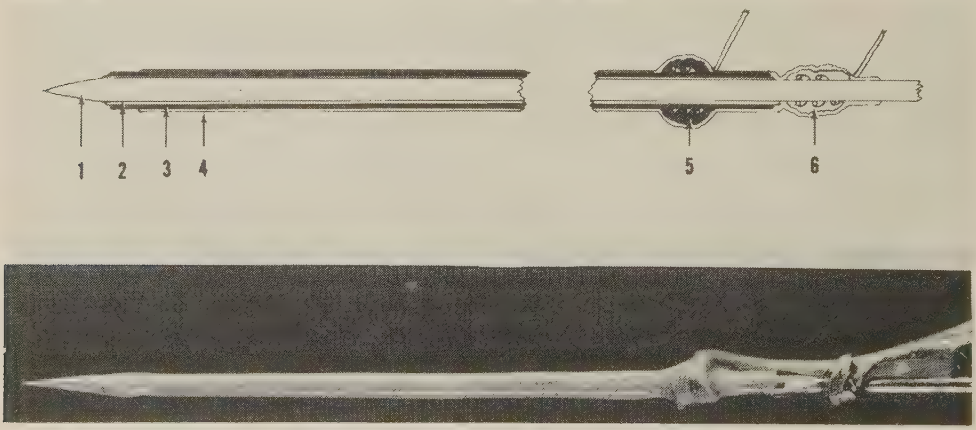

FIG. 1. Top diagram shows a cross-sectional view of construction of bipolar silver paint electrode. Key: 1) Steel core. 2) Epoxylite. 3) Printed circuit paint coating. 4) Epoxylite coating. 5) Lead wire joint of second pole (silver paint pole). 6) Silver solder joint of first pole on steel core. Bottom photograph shows completed bi-polar silver paint electrode.

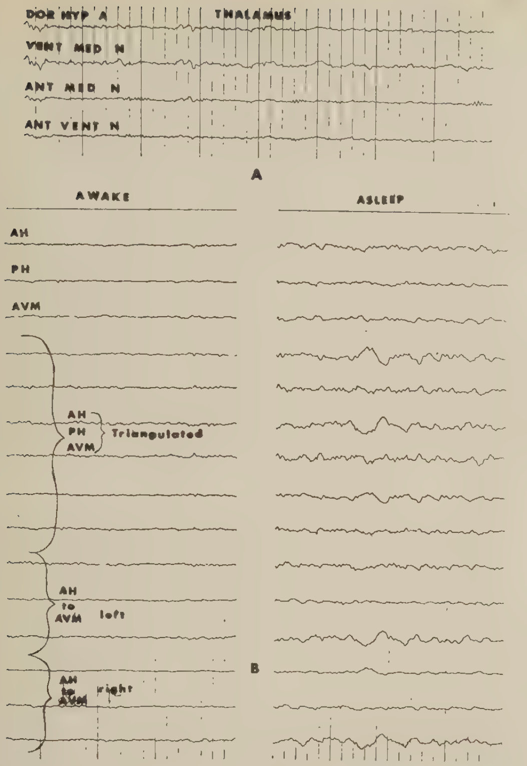

FIG. 2. E.E.G. tracings made from multi-polar silver paint electrode imbedded in posterior thalamic areas (A). The most proximal recording point was located in anterior ventral thalamic nucleus, Ant Vent N; the second recording point, in anterior medial thalamic nucleus, Ant Med N; the third point, in ventral medial thalamic nucleus, Vent Med N; the most distal recording point was located in dorsal hypothalamic area, Dor Hyp A. (B) Pairs of bipolar silver paint electrodes implanted in anterior hypothalamic area (AH); in posterior hypothalamic area (PH); and in anterior ventral mesencephalic reticular formation (AVM).

as Lubriseal. The Lubriseal was allowed to cover the already exposed tip of the steel core and as much of the first silver paint layer as is desired for a stimulating or recording surface (Fig. 1). This grease coating prevents the insulating coat from covering the exposed recording and/or stimulating surfaces. It can be removed later by gently wiping the tip with xylol soaked cotton. 7) The electrode was again run through the Epoxylite dipping procedure (Step 3) and 3 to 4 coats were applied making certain that these layers were carried over the previously made lead off wire joints. The electrode was then returned to the oven at a reduced temperature of 20°C to prevent contraction of the Tensolite insulation on the lead off wires. It is best to allow this coat to harden in the oven for at least 48 hours. Steps 5, 6 and 7 can be repeated for as many poles as desired.

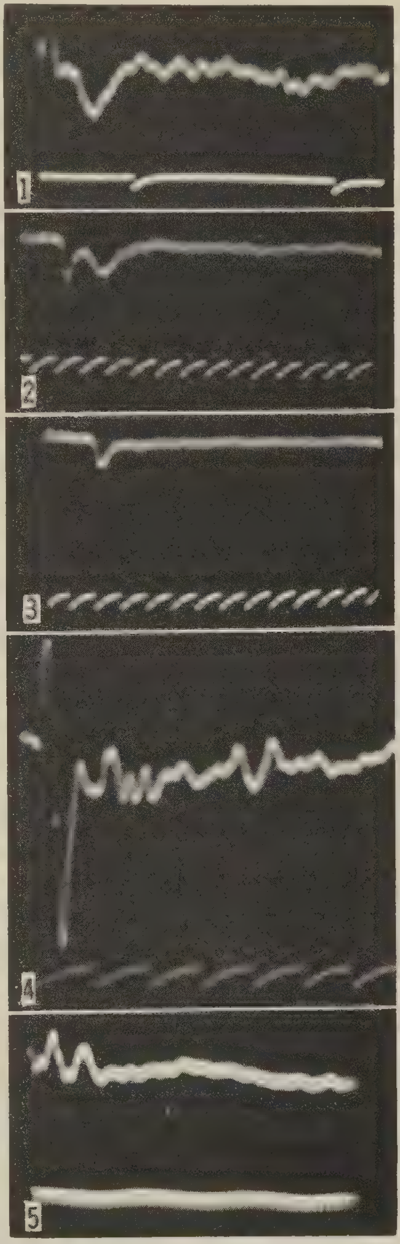

FIG. 3. Oscillographic recordings made from bipolar silver paint electrodes implanted in (1) medial longitudinal fasciculus; (2) sulco-marginal bundle of spinal cord on same side as stimulating electrode; (3) sulco-marginal bundle of spinal cord on opposite side from stimulating electrodes; (4) anterior gray horn of spinal cord; (5) vestibulo-cerebellar pathway.

Results. Generally, it was found that electrodes made according to the above procedure were of relatively low impedance. The silver paint poles were of somewhat higher impedance than the steel silver solder coated core. For example, in a direct current system and using isotonic saline as a conductor, impedance of the steel core pole was about 3,000 ohms while that of the silver paint pole was about 30,000 ohms. This may appear to be high; however, in animal bio-electric systems (*in vivo*) the characteristics are such that passage of current and voltage indicated a much lower impedance than that found in D.C. systems.

Deep electroencephalographic tracings were made from bi-polar silver paint electrodes implanted chronically in the anterior hypothalamus, posterior hypothalamus and the basal-anterior mesencephalic reticular formation of the cat (Fig. 2). Tracings were also taken from the thalamus by a multipolar electrode (4 separate poles) (Fig. 3). One notes that the recordings are stable and free of electrode "noise." Further the electroencephalograph must be attenuated at the pre-amplifier to reduce the sensitivity of the electrode.

Using both bi-polar and multi-polar electrodes in stimulating and recording sites of the brain stem, oscillographic recordings were made in the vestibular nuclei, the medial longitudinal fasciculus, the sulco-marginal bundle and anterior gray horns of the spinal cord. Stimulating voltages of from 0.1 of a volt to 1.0 volt were used in most cases. Clear cut potentials were easily obtained at the above low voltages in the vestibulo spinal system (Fig. 3).

Electrodes in the foregoing positions have been implanted chronically in cats and left in position for up to 5 months without dramatic changes in either the electroencephalographic or oscillographic tracings. Changes do occur in the above recordings for about 2 weeks following implantation. This is probably due to a chemical change on the exposed silver recording and stimulating points thru action of extracellular fluid, because a light film of blackened silver could be scraped from the recording and stimulating points on removal from the brain.

Acute short term experiments have been performed with these electrodes with equally good results.

Summary. A new type of multi-polar stimulating and recording electrode was developed to answer certain technical difficulties arising through use of standard type electrodes. This relatively low impedance electrode was constructed by using silver "printed circuit" paint over coats of insulating material without greatly increasing the diameter of the electrode core. Both acute and chronic bio-electric experiments were carried out employing the above electrodes giving good amplitude, stable electroencephalographic and oscillographic records from the central nervous system.

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Passage of Barbital into Cerebrospinal Fluid.* (25610)

LESTER C. MARK, LEONARD BRAND, PETER G. DAYTON, DOLORES TALLER AND
E. M. PAPPER

Dept. of Anesthesiology, Columbia-Presbyterian Medical Center and N. Y. University Research Service, Goldwater Memorial Hospital, N. Y. City and Laboratory of Chemical Pharmacology, Nat. Heart Inst., N.I.H., Bethesda, Md.

Previous studies have shown that barbital given intravenously passes slowly into the brain(1,2). In this report, the latter study was extended to include observations in dogs on passage of barbital into the cerebrospinal fluid.

Methods and materials. Barbital was determined by the method of Giotti and May-

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TABLE I. Sodium Barbital 100 mg/kg Intravenously to Dogs.

Time, min.	Wt, kg	Plasma, mg/l*	Brain, mg/kg*	CSF, mg/l*	Conc. ratio	
					Brain/plasma	CSF/plasma
3	10.2	189	52	27	.27	.14
”	10.8	”	44	12	.23	.06
”	10.0	169	27	13	.16	.08
9	11.4	162	51	35	.31	.22
”	13.2	130	58	38	.45	.28
30	23.0	151	93	64	.62	.43
”	24.5	141	99	68	.70	.48
”	19.5	107		43		.40
60	10.0	129	104	98	.80	.76
”	9.0	133	95	105	.72	.79
180	9.1	82	69	76	.85	.93
”	22.0	114	91	105	.80	.92

* Concentrations expressed as mg/l or mg/kg of barbital acid.
Values corrected for 90% recovery.

nert(3). For analysis of the compound in brain (cerebral hemispheres) 2 ml of a homogenate containing 0.5 g of tissue were used. The method gave negligible blank values and recoveries averaged 90%. Sodium barbital (100 mg/kg) was given intravenously as a 10% neutral solution to dogs over a period of 5 seconds. After various time intervals, the animals were sacrificed by rapid intravenous injection of 4 to 6 ml of a saturated solution of potassium chloride, which produced cardiac standstill within 5 seconds. Heart blood was immediately obtained by cardiac puncture. Cerebrospinal fluid was withdrawn from the cisterna magna and the brain was then removed for analysis of its barbital content.

Results. Barbital concentrations in the plasma were lower the longer the time interval after drug administration (Table I). Brain/plasma concentration ratios, low at first, reached a maximal value in about 60 minutes. Concentration of barbital in the cerebrospinal fluid rose more slowly than that in the brain (Table I). Cerebrospinal fluid/plasma ratios were low at 3 minutes but became progressively higher reaching unity at 3 hours.

Discussion. Barbital distributes uniformly in total body water; consequently accumulation of the drug in any tissue is limited by amount of water present in that tissue. Since the water content of the brain is about 80% (4) concentration of barbital in brain should eventually be about 80% of that in plasma.[†]

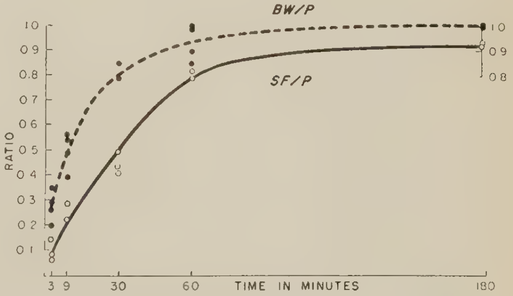

FIG. 1. Brain water/plasma (*BW/P*) and cerebrospinal fluid/plasma (*SF/P*) concentration ratios for barbital given intrav. to dogs. Values obtained from Table I and from previously published(2) experiments, in which only brain water/plasma ratios were determined. Superimposed points are not differentiated.

At equilibrium brain water/plasma concentration ratio should approximate unity. This value was reached in about 60 minutes (Fig. 1). Since cerebrospinal fluid is practically an ultrafiltrate of plasma, at equilibrium the ratio of barbital concentration in cerebrospinal fluid to that in plasma should likewise approximate unity. These results show that there is a barrier to passage of barbital both from blood into brain and from blood into cerebrospinal fluid. The barriers may be similar. The observation that barbital appears in the cerebrospinal fluid even more slowly than in the brain, suggests that the drug may pass from blood into brain and then into cerebrospinal fluid as well as by direct passage from blood into cerebrospinal fluid. The relative contribution of these routes to entrance of barbital into the cerebrospinal fluid cannot be stated now.

[†] Barbital is not appreciably bound to plasma proteins(5).

In contrast to the slow passage of barbital into brain and cerebrospinal fluid, the more lipid soluble thiopental passes rapidly into the central nervous system: maximal concentrations appear in the brain almost immediately after intravenous injection(6) and in the cerebrospinal fluid within 7 minutes(7).

Summary. Experiments in dogs have shown that barbital passes slowly into the brain and even more slowly into cerebrospinal fluid. It is possible that the blood cerebrospinal fluid barrier and blood brain barrier are similar for this drug.

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Erythrocyte Extract (Hemolysate) and Tissue Thromboplastin: Their Action on Heparin Activity.* (25611)

ARMAND J. QUICK AND MARY ELIZABETH HICKEY
Dept. of Biochemistry, Marquette University School of Medicine, Milwaukee

Heparin with its co-factor, which originally was designated albumin X, is a powerful antithrombin(1). By means of certain agents such as protamine(2), toluidine blue(3) and polybrene(4) (a poly 1,5 dimethyl-1,5-diazoundecamethylene methobromide), heparin is inactivated directly and, presumably, stoichiometrically. Tissue thromboplastin also inactivates heparin activity, but from results of earlier studies(5), it was concluded that the anti-heparin action of thromboplastin is indirect, brought about by increase in rate and amount of thrombin produced, thereby exceeding the antithrombin activity of heparin. In the present study an attempt is made to determine the nature of the antiheparin action of erythrocyte extract (hemolysate)(6,7,8). For comparison the antiheparin activity of tissue thromboplastin was restudied.

Materials and methods. Preparation of platelet-poor human native plasma. Blood collected with silicone-coated syringe and needle was transferred to heavy walled test tubes coated with silicone. After chilling in ice bath for 20 minutes, blood was centrifuged for 30 minutes in a Servall Superspeed centrifuge kept in cold room (4-6°C) and the plasma carefully removed with a silicone-coated pipette. *Erythrocyte extract*(9). Human erythrocytes freed from platelets and leucocytes by repeated washing and differential centrifugation were suspended in a volume of physiological saline equal to that of blood from which they were obtained. Complete hemolysis was obtained by freezing at –20°C and then thawing. *Heparin.* One hundred mg sodium salt in 10 ml (Lederle). *Protamine sulfate.* Nutritional Biochemicals Corp. product was dissolved in distilled water. *Prothrombin consumption time with added hemolysate.* In the basic test as previously described(1), 1 ml of native platelet-poor plasma is mixed with a fixed amount of hemolysate and incubated at 37°C. Fifteen minutes after solid clot has formed, it is removed by wrapping it on to a wooden applicator. After 45 minutes of further incubation at 37°C, prothrombin of the serum is determined by the one-stage technic using rabbit plasma adsorbed with $Ca_3(PO_4)_2$ as source of fibrinogen

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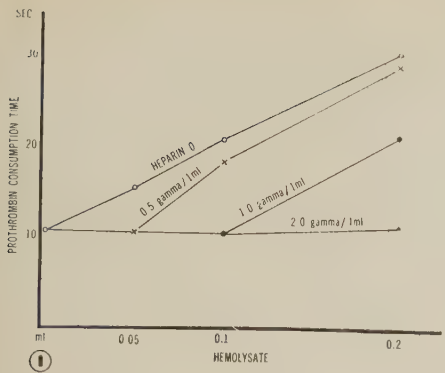

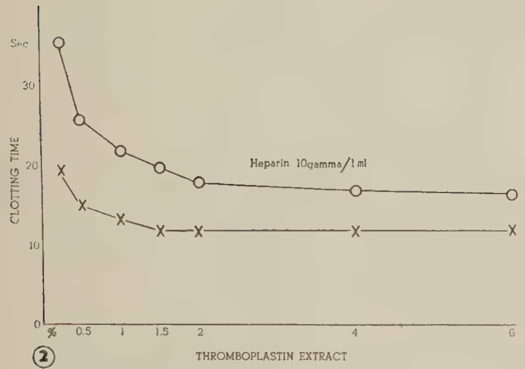

FIG. 1. Effect of increasing amounts of hemolysate on heparin activity as measured by prothrombin consumption test.

FIG. 2. Influence of heparin on prothrombin time of human plasma with varying concentrations of tissue thromboplastin (rabbit brain extract).

and labile factor. In the present investigation, the test was carried out in silicone-coated test tubes. The agents, heparin and protamine sulfate, which were studied were mixed with native plasma before hemolysate was added. *Prothrombin time.* The one-stage technic as previously described was employed (10). Thromboplastin was prepared from rabbit brain by the acetone dehydration method. Suspensions of thromboplastin varying from 0.25 to 6% in saline were incubated at 50°C for 20 minutes.

Results. Effect of heparin on prothrombin consumption time of native platelet-poor plasma with added hemolysate. Native platelet-poor human plasma fails to clot in silicone at 37°C. On adding hemolysate, clotting occurs and prothrombin consumption time increases in direct proportion to amount added (Fig. 1). As little as 0.5 μg of heparin/ml of plasma usually inhibited completely consumption of prothrombin resulting from addition of 0.05 ml of hemolysate. On increasing the quantity of hemolysate, consumption of prothrombin is resumed and when prothrombin consumption is plotted against amounts of added hemolysate, a straight line, fairly parallel to the control curve without heparin, is obtained. When concentration of heparin is 2 μg/ml of plasma, little or no consumption of prothrombin occurs, even when 0.2 ml of hemolysate is added.

Effect of protamine sulfate on heparin action in prothrombin consumption test with added hemolysate. When plasma contains 1 μg of heparin/ml, no consumption of prothrombin occurs on addition of 0.05 ml of hemolysate until concentration of protamine sulfate is 2-3 μg/ml of plasma (Table I). In the absence of added heparin, protamine sulfate does not influence the effect of hemolysate on consumption of prothrombin.

Effect of heparin on prothrombin time with varying amounts of thromboplastin. Prothrombin time of normal plasma is progressively decreased by increasing quantities of tissue thromboplastin (rabbit brain) until a minimal constant value of 12 seconds is obtained. Concentrations of thromboplastin greater than 6% may have a slightly inhibitory effect.

When 10 μg of heparin are added to 1 ml of oxalated plasma, and a series of prothrombin time determinations made using same concentrations of thromboplastin as in the control, a similar curve but with longer clotting time values is obtained (Fig. 2). As in the control, a constant value is obtained which is not shortened by excess thromboplastin.

Discussion. The clotting factor in erythrocytes corresponds chemically to a phospholipid(11). It possesses the unique property of causing a high consumption of prothrombin when added to native platelet-poor human plasma in a silicone-coated test tube(12), whereas a platelet extract causes no measurable consumption of prothrombin under similar conditions(13). This suggests that the clotting factor, erythrocytin, is not identical with the clotting factor in platelets which functions in the generation of thromboplastin

TABLE I. Inactivation of Heparin by Protamine Sulfate.

Heparin,* μg	Protamine sulfate, μg	Hemolysate, ml	Prothrombin consumption, sec.
			12
1		.05	12.6
1	1	"	12.6
1	2	"	17.2
1	3	"	26
1	5	"	26
	5	"	24
		.05	26
		.1	31
		.2	40
.5		.05	12
"		.1	12
"		.2	31
"	10	.05	26
"	"	.1	31
"	"	.2	46

* Heparin was mixed with 1 ml of platelet-poor normal native human plasma in a silicone-coated test tube. Protamine sulfate was then added, followed by the hemolysate.

even though both can participate in production of intrinsic thromboplastin.

Heparin and hemolysate have a mutually antagonistic action on prothrombin consumption. As little as 0.5 μg of heparin/ml of plasma completely inhibits action of 0.05 ml of hemolysate. On increasing the quantity of hemolysate, inhibitory activity of heparin is sufficiently overcome to allow consumption of prothrombin and a proportionality between amount of hemolysate and the prothrombin consumption occurs. However, even when the amount of hemolysate is quadrupled, heparin activity is not completely abolished as it is when heparin is neutralized by protamine sulfate (Table I). When concentration of heparin is increased above 2 μg/ml of plasma, complete depression of prothrombin consumption results.

Two explanations for the antiheparin action of hemolysate seem probable: either hemolysate neutralizes heparin directly as does protamine sulfate, or hemolysate acts by participating in production of thromboplastin which brings about generation of sufficient thrombin to overcome antithrombic activity of heparin. Since hemolysate does not completely overcome the heparin effect and fails to cause consumption of prothrombin when concentration of heparin is above 2 μg/ml of plasma, the second explanation appears to be the more likely.

Generation of intrinsic thromboplastin is a complex reaction. It not only requires adequate quantities of a platelet or erythrocyte factor, thromboplastinogen (factor VIII), PTC (factor IX) and perhaps other primary agents, but it also depends on an autocatalytic reaction which is probably mediated through thrombin(13). Presumably, prompt neutralization of thrombin as it is formed effectively blocks the clotting reaction. By carrying out the reaction in a silicone-coated test tube, the catalytic action of a glass surface is eliminated and sensitivity of the test to minute amounts of heparin is greatly increased.

Protamine sulfate neutralizes heparin completely. Approximately 2-3 μg are required to inactivate 1 μg of heparin. Significantly, addition of protamine sulfate to normal native plasma does not alter prothrombin consumption resulting from a fixed amount of hemolysate. Since the method is sensitive to amounts of heparin less than 0.5 μg/ml, it appears unlikely that normal plasma contains a significant quantity of free or active heparin.

The prothrombin time curves in Fig. 2 suggest the nature of antiheparin action of tissue thromboplastin. If a direct or true inactivation of heparin occurred, prothrombin time of plasma containing heparin should become 12 seconds at high concentrations of thromboplastin. A more likely explanation is an indirect inactivation. The thrombin produced exceeds the antithrombin capacity of heparin.

If the antiheparin action of hemolysate and of tissue thromboplastin is indirect and depends on generation of thrombin, it can be concluded that this is not a specific property but a general one possessed by any material which has thromboplastic activity or takes part in the generation of intrinsic thromboplastin. Since it has not been established that heparin has an essential role in maintaining fluidity of blood, and since clinical evidence has been found that even marked hyperheparinemia may produce only a mild bleeding state (14), antiheparin activity may perhaps be merely incidental and have little physiological significance.

Summary. The inhibitory action of heparin on prothrombin consumption of normal platelet-poor human plasma in a silicone-coated test tube can be partially overcome by increasing amounts of hemolysate. Likewise, prothrombin time of human plasma to which a fixed amount of heparin has been added can be shortened and brought to a constant but higher value than normal by adding increasing amounts of tissue thromboplastin.

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Quantitative Relationship of Osteoclasts to Parathyroid Function.* (25612)

R. J. TOFT AND ROY V. TALMAGE
Dept. of Biology, Rice Inst., Houston, Texas

Problems concerning the role of osteoclasts in bone dissolution have long been investigated. Numerous studies have attempted to clarify this basic relationship, particularly in reference to the part played by osteoclasts in parathyroid hormone effect on bone resorption. These earlier studies have been reviewed by Hancox(1,2). Of particular interest are reports of Selye(3), which referred to increases in numbers of osteoclasts after parathyroid extract administration, and of Bloom *et al.*(4), which demonstrated qualitative increases in osteoclasts coupled with decreases in numbers of osteoblasts during laying cycle of pigeons. More recently Gaillard(5) noted significant increases both in number and activity of osteoclasts in incubated foetal bone, under the influence of a substance elaborated from concurrently incubated parathyroid tissue, or from parathyroid extract added to the media. Talmage *et al.*(6) were able to show qualitative increases in numbers of osteoclasts in long bones of rats undergoing continuous peritoneal lavage with calcium- and phosphate-free rinse. The only attempt to use quantitative determination of osteoclast concentrations as an index of metabolic activity has been the recent work by Myers *et al.*(7) on the mandibular condyle. The purpose of our study was to develop a method for quantitation of osteoclastic material which could be used as index of bone dissolution and of parathyroid activity.

Materials and methods. These experiments utilized approximately 75 male Holtzman rats with weight range restricted to between 225 and 250 g. All were placed on a calcium- and phosphate-free diet for 24 hr before start of each experiment. Bilateral nephrectomies and parathyroidectomies were performed under ether anesthesia. Continuous peritoneal lavage was carried out by introducing into the peritoneal cavity, through a stainless steel indwelling plug(8), 30 ml of a solution containing 0.8% NaCl and 0.5% glucose at pH 7.4. After equilibration for 1 hour, this fluid was removed and immediately replaced with another 30 ml of lavage solution. Animals were

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* Aided in part by grant from Atomic Energy Comm.

not anesthetized during lavage. Commercially available parathyroid extract,[†] when administered, was given intraperitoneally. *Histological procedures.* When animals were sacrificed, distal portion of the femur was exposed and the bone snapped at the epiphysis. Remainder of femur was sawed across the shaft one-fourth inch from the metaphysis. This distal segment, which included subepiphyseal and portions of the trabecular area, was fixed for 8 to 12 hr in Helly's fluid. After washing in water, bones were decalcified with several changes of 5% formic acid for several days. Following dehydration and infiltration with celloidin, they were embedded in paraffin and sectioned transversely at 7 μ. The area examined was that just beneath deepest penetration of epiphyseal plate into the diaphysis. This area represents, in all animals, the most uniformly growing area. Further, this technic permitted preparation of sections of each bone at exactly the same area of bone to insure maximum uniformity of conditions. Since quantitative counts of osteoclasts were to be made, a staining procedure was sought which would permit easy identification of these cells. After staining with Ehrlich's hematoxylin, slides were placed in a mixture of Biebrich scarlet and Orange II. Following treatment with phosphotungstic mordant, they were stained with Fast Green FCF, dehydrated and cleared. This procedure caused osteoclasts to appear as magenta colored cells in contrast to bone matrix which stained green and to erythrocytes which stained orange. For quick identification of osteoclasts this stain proved markedly superior to hematoxylin and phloxin tried earlier. *Counting technic.* By an ocular grid in binocular microscope at magnification of 430 ×, an area of bone 0.26 mm^2 was contained within the field of the grid. The entire cross-section of each bone was scanned and osteoclasts counted. Results were expressed as number of osteoclasts/field. Due to large size of an osteoclast, it was not routinely possible to determine which segments noted in the microscopic field made up a single osteoclast. Therefore, portions of osteoclasts which appeared as separate units in the field were counted separately. While this method does not give an absolute count of osteoclasts in a given area, it permits a quantitative representation of amount of osteoclastic material in a given volume of tissue.

TABLE I. Effects of Various Stimuli on Osteoclast Concentration in Rat Femurs.

Group		No. rats	Osteoclasts/field*
1	Normal, control	32	3.55 ± .07
2	Normal, 250 USP PTH†	4	4.77 ± .44
3	PTX‡ 7 days	4	2.85 ± .18
4	PTX 7 days, 50 USP PTH	4	3.42 ± .49
5	NEPX§ 24 hr	16	6.95 ± .32
6	LAV‖ 12 "	9	4.54 ± .48
7	LAV 32 "	5	6.09 ± .18

* Field size 0.26 mm^2.

† Parathyroid extract given 18 hr before sacrifice.

‡ Parathyroidectomized.

§ Nephrectomized.

‖ Continuous peritoneal lavage.

Groups 2 and 3 are statistically different from Group 1 ($P = < .001$).

Results. In Table I are summarized results of a variety of experiments designed to show a quantitative relationship between parathyroid stimulation and osteoclast formation. The ratio found in animals maintained on stock diet served as the norm. Since 2 or 3 days on calcium- and phosphate-free diet did not significantly affect this norm, animals so treated were grouped with controls. Parathyroidectomy for 7 days reduced significantly the osteoclast count. Treatment of such animals with 50 USP units of parathyroid extract returned count to normal, as determined by bones removed 18 hr after hormone administration. Quantitative increases in osteoclast count above normal were produced by treatment with 250 USP units of extract, by technic of peritoneal lavage, and by nephrectomy. Degree of stimulation noted was maximum in bones taken from rats nephrectomized for 24 hr or from those undergoing continuous peritoneal lavage for 32 hr. Parathyroid extract (250 USP units) produced a stimulation in bones, removed 18 hr after administration, similar to that found in bones removed after only 12 hr of continuous lavage.

[†] Eli Lilly Co., Parathyroid Extract.

A further study was made to determine if the ratio of cytoplasmic to nuclear material in these multinucleated cells varied with degrees of parathyroid stimulation studied. Counts of nuclei/osteoclast, however, remained constant even in bones showing a 100% variation in osteoclast count. The fact that number of nuclei/osteoclast remains constant, even though concentration of osteoclasts can change greatly, lends support to the idea that osteoclasts are formed by coalescence of other cell types; namely, osteoblasts and osteocytes.

Discussion. Our purpose was to determine if changes in parathyroid activity could be detected by quantitative change in osteoclast concentration of a selected area of long bone. Our data indicate such to be the case and also demonstrate that the procedure establishes a reliable index of changes in endogenous production of parathyroid hormone as well as those noted by exogenous administration.

The data also are of interest in regard to normal functioning of the parathyroid gland. According to work of McLean(9) and recent report of Talmage *et al.*(10), bone is under continuous influence of hormone from parathyroids due to necessity of "feeding back" calcium into body fluids. This concept is further substantiated by above data which indicate that normal osteoclast concentration can only be maintained in parathyroid-intact animals, and that a significantly lower level appears following parathyroidectomy.

A further point of interest is substantiation of hyperactivity of parathyroids following nephrectomy. This has been reported in studies of histological changes in the gland produced by nephrectomy(11,12). Degree of hyperactivity can be compared to animals lavaged continuously for 32 hr with a calcium- and phosphate-free rinse in which it can be calculated(13) that over 50 mg of calcium were removed from the animal. However, evidences of secretory changes in parathyroid tissue are most difficult to produce by means other than nephrectomy, and it is hoped that our technics will serve as a valuable aid in establishing physiological control of parathyroid secretion.

Summary. A method for quantitative determination of amount of osteoclast material in rat femurs is presented. The number of osteoclasts/unit area is sufficiently constant in normal animals to permit statistical differentiation between these and experimental groups. Parathyroidectomy reduced the osteoclast count, which was subsequently returned to normal by parathyroid extract administration. Nephrectomy, continuous peritoneal lavage, and parathyroid extract administration to normal animals, all increased significantly the number of osteoclasts/unit area. Maintenance of a normal osteoclast concentration above that of parathyroidectomized animals is discussed in relation to McLean's "feed-back" principle of parathyroid action under which continuous secretory activity of these glands is assumed. It is felt that determination of osteoclast concentrations as described can be used as index of parathyroid activity in studying problems related to parathyroid secretion.

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Deposition of Cholesterol in Experimental Rabbit Atherosclerosis.* (25613)

Erwin Schwenk and Dean F. Stevens (Introduced by Gregory Pincus)
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

The following investigations were made to study the fate of cholesterol administered to rabbits in amounts known to effect atherosclerotic lesions.

Methods. Several groups of rabbits were fed commercial cholesterol (Wilson), each animal receiving 900 mg/day for 12 weeks. In each group the commercial material was replaced for 6 days as outlined below by one or the other of 2 types of radioactive material. Cholesterol-C^{14}, obtained from livers of rats injected with sodium acetate-1-C^{14} for various purposes, was purified through the digitonide and the dibromide. Cholesterol-H^3 was prepared by Wilzbach's method at New England Nuclear Corp. from cholesterol acetate and tritium and was stabilized in this laboratory by boiling several times with methanolic sodium hydroxide until the last operations showed a nearly constant count of 3.196×10^6 min./mg. This material was dissolved in boiling methanol with calculated amounts of commercial cholesterol (Wilson) and left to crystallize in the cold. It could not be purified further through the dibromide because repeated treatment of a sample with bromine and afterwards with zinc and acid showed a continuous loss of radioactivity. Cholesterol was dissolved in ether and poured together with 5% cotton oil over the rabbit chow and ether was eliminated by heating under stirring or shaking on a water bath. Feeding of radioactive and commercial material was done every day except on Sunday. Young healthy male and female Dutch belted rabbits weighing 3 to 4 lb were arranged in groups of 10 to 13 animals so that each group contained an equal number of males and females. One group was fed cholesterol-H^3 during the first week, another during seventh week and one during twelfth week. In another series cholesterol-C^{14} was fed during the first week and to 3 animals for 2 days in the twelfth week. At end of the week of radioactive cholesterol feeding, 3 or 4 animals were killed and worked up, while remainder of group was continued on ordinary cholesterol, until at end of 12 weeks all animals were killed.

The animals were killed by heart puncture and blood was taken for cholesterol determinations. The animals were then dissected into aortae, livers, small intestines, adrenals, brains, kidneys and blood; the corresponding tissues for each group were combined, weighed and hydrolyzed with 30% KOH and the cholesterol isolated as digitonide. In the C^{14} experiments this material was further purified by bromination. All these operations have been described(1). Cholesterol-H^3 was counted in a scintillation counter, while for cholesterol-C^{14} an end window or scintillation counter was employed.

All animals showed atherosclerotic lesions of varying degrees on macroscopic inspection but no histological evaluation was made. Cholesterol determination in blood serum at time of killing showed usual considerable rise of serum cholesterol in most animals.

Results. The results are presented in Tables I and II. Radioactive cholesterol was found at end of 1 week feeding in each tissue but in different amounts. The brain showed lowest deposition. Even after only 2 days feeding of cholesterol-C^{14} as in Exp. 3 of Table I the substance isolated from aortae contained radioactivity. From the count of this material and total radioactivity fed, one can calculate the amount of cholesterol laid down during feeding and relate it to weight of tissue. It is thus found that the amount of cholesterol deposited in the aorta/feeding day/animal/g of wet aorta weight was 0.03 mg (0.029-0.032) for the 3 experiments carried out with cholesterol-C^{14}. Exp. 2 showed that after deposition of radioactive substance in the aorta, further feeding of ordinary cholesterol not only covered the radioactive layer but replaced the radioactive substance and a nearly

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complete loss of it took place during the following 11 weeks. This disappearance of radioactivity cannot be explained by mere dilution of the active substance *in situ* because at the end of, for example Exp. 2, the aortae had increased their cholesterol content from 2.5 mg to 5.4 mg/g wet tissue, while radioactivity decreased from 130 counts to a trace. A similar picture was obtained for all other tissues. In almost all tissues purification of the

TABLE I. Feeding of Cholesterol-C^{14} and of Commercial Cholesterol to Rabbits.

Exp. No.	Cholesterol-C^{14}		Aorta		Liver		Small intestine		Adrenal		Kidney		Blood		Brain		Counts/min./mg in cholesterol-C^{14} fed
	Fed week	Killed week															
1	1	1	1.9	702	6.7	1152	1.28	937	33.3	536	3.3	578	1.5	1078	9.4	14	1768
				604		1126		1011		368		656		1127		12	
2	1	1	2.5	130	2.7	365	2.3	373	43.1	217	2.9	185	1.0	312	13	3	473
				246		297		317		270		138		308		3	
	1	12	5.4	trace	7.7	trace	.6	trace	45.9	trace	2.6	trace	1.8	trace	5.0		
3	*	12	6.4	39		57		355									1392
				25		40		359									

In each column figure on left side represents mg cholesterol-C^{14} isolated/g wet tissue. The upper figure on right side gives counts/min./mg in isolated digitonin cholesterol-C^{14} and the lower figure the corresponding counts after bromination treatment.

* In this experiment cholesterol-C^{14} was fed for last 2 days only.

TABLE II. Feeding of Cholesterol-H^3 and of Commercial Cholesterol to Rabbits.

Exp. No.	Radioactive cholesterol		Aorta		Liver		Small intestine		Adrenal		Kidney		Blood		Brain		Counts/min./mg in cholesterol-H^3 fed
	Fed week	Killed week															
1	1	1	1.5	2410	2.8	16880	2.4	18960	35.6	10805	2.8	6266	.4	11550	13.4	0	29500
	1	12	3.0	208	7.4	141	3.0	68	32.1	190	4.3	122	2.8	105	8.3	102	
2	7	7	2.9	1250	8.9	6313	.8	5475	39.7	1472	2.3	2038	1.4	4094	18.2	56	11800
	7	12	2.5	1695*	4.4	1152	.6	803	109	2434*	1.3	1369	2.1	981	8.0	127	
3	7	7	3.0	960	6.7	2057	.95	5203	24.2	1515	3.7	1382	3.3	2969	10.9	39	9155
	7	12	1.63	862	4.95	1039	1.19	756	57.2	1291	2.21	898	.31	865	9.92	56	
4	12	12	3.1	2900	15.4	4610	1.2	15030	62.8	1372	2.2	3615	2.5	8160	9.0	72	29500

In each column figure on left represents mg cholesterol-H^3 isolated/g wet tissue. Figure on right gives counts/min./mg in the isolated cholesterol-H^3.

* These figures were assumed to be due to an error and the experiment was repeated as Exp. 3.

digitonin precipitate by bromination showed some formation of cholesterol derivatives, which were still precipitable.

Much the same situation was found in Exp. 1-4 of Table II with cholesterol-H^3. When this substance was fed in the first or seventh week and ordinary cholesterol was given during remaining part of experiments, radioactivity at the end of 12 weeks decreased considerably in all tissues. As in experiments with C^{14} this decrease was greater than a possible dilution caused by increase of total cholesterol in the tissues. For example, in Exp. 1 the aorta cholesterol doubled from 1.5 to 3.0 mg/g wet tissue whereas the count dropped from 2410 to 208. In other tissues such as liver, small intestine or adrenal, this discrepancy, possibly because of their specific metabolism, was more pronounced. Only in the brain was a definite increase of radioactive material observed at end of experiments, although these counts remained quite low. This may be so because the brain has apparently a very low cholesterol turnover—adult brain does not synthesize cholesterol from acetate(2)—and exchange of cholesterol between blood and brain may be very limited(3). The decrease in counts of cholesterol in all tissues is therefore determined not only by dilution with non-radioactive cholesterol present in the tissue prior to feeding, or by subsequent deposition of non-radioactive substance fed after the radioactive feeding period, but also by the specific metabolism including biosynthesis of cholesterol in each tissue. It is further interesting to note that even after one week's feeding whether it was the first, seventh or twelfth week in the experiment, the counts of cholesterol-H^3 used were diluted in each tissue at a different rate. Thus for example the relation of the count in starting cholesterol to the count in cholesterol isolated from the aortae in Table II is 8.2, 10.6, 10.5 and 9.8 after 1 week of feeding of radioactive material. The same calculation however gives 36.4 and 25.1 in Exp. 1 and 2 (Table I) with cholesterol-C^{14}, which were carried on for 1 week. It was pointed out above that amount of cholesterol laid down in the aortae/day/animal, and /g of wet aorta weight can be calculated as 0.03 mg. The same calculation extended to experiments with cholesterol-H^3 in Table II, gives considerably lower values, namely 0.011 (0.005-0.017). Similar differences are found between experiments of Tables I and II for other tissues investigated, with exception of the brain where the figures are too small to allow evaluation. Dilution of the original count was always greater for cholesterol-H^3 than for cholesterol-C^{14}. In both types of experiments however the amount of radioactive material fed was the same, namely 900 mg/day/animal and it is therefore evident that the amount of deposited radioactive material must be the same for each corresponding pair of tissues of Tables I and II. The explanation of the discrepancies seems to be that in the C^{14}-material the radiocarbon is part of the skeleton of the molecule, while the radioactive hydrogen is on its periphery, exposed to attack of oxygen, and therefore can be detached from the molecule without destroying its integrity. Borgstroem and Wintersteiner(4) have indeed shown that cholesterol in colloidal suspension, under conditions similar to those in blood, is very easily oxidized by molecular oxygen, giving 7-keto- and 7α- or 7β-hydroxycholesterol. Katienagar and Morton(5) found in plaques of human aortae Δ^3,Δ^5-diene-7-one and other substances derived from cholesterol by oxidation. These findings were confirmed by Henderson(6) who by paper partition chromatography demonstrated in human aortae the presence of Δ^5-cholestene-$3\beta,7\beta$-diol, Δ^5-cholestene-$3\beta,7\alpha$-diol, cholestane-$3\beta,5\beta,6\beta$-triol and Δ^5-cholestene-3β, 24 (or 25)-diol. All these substances can only be formed from cholesterol by loss of hydrogen which in cholesterol-H^3 causes the observed decrease of radioactivity. A further loss of hydrogen and therefore tritium may be effected by dehydrogenation. Festenstein and Morton(7) made probable the presence in tissues of a specific dehydrogenase, the normal substrate of which is cholesterol.

This peripheral oxidation of cholesterol may account for the striking differences between experiments with the 2 types of radioactive cholesterol (*i.e.*, C^{14} or H^3). However the almost complete disappearance of radioactivity when one week of radioactive cholesterol is followed by 7 or 11 weeks of ordinary cholesterol, is explained by degradation of the cholesterol skeleton itself in formation of bile acids and by elimination of cholesterol through the feces.

The figures for cholesterol isolated from blood suggest that a large part of oxidative degeneration must occur in blood, in agreement with experiments of Borgstroem and Wintersteiner(4). The small differences between the digitonin and dibromo-cholesterol figures in Table I showed that in the course of 1 week during which radioactive material was fed, little degradation of the cholesterol skeleton itself occurred.

Continuation of experiments with both types of radioactive cholesterol by feeding non-radioactive substance resulted in considerably lower counts in most tissues. This shows that there is a continual exchange and subsequent degradation taking place between cholesterol of tissues and blood. Such reaction has been observed before(8) but it is surprising that in the aortae, where cholesterol is deposited in plaques, such an exchange is also functional. While it is not possible to decide how much of these changes occur in blood before and how much in the tissue after deposition of cholesterol, it is quite probable that almost all of it is finally broken down in the liver and so disposed of, thus explaining disappearance of radioactivity at end of experiments.

The experiments here reported are in agreement with Biggs and Kritchevsky(9) who fed cholesterol-H^3 to rabbits and established that exogenous cholesterol is deposited in tissues, and of Dayton(10) who fed cholesterol-C^{14} to chicks. The present work however shows that cholesterol, once deposited, is continuously exchanged between tissues including aortae and blood and that the amounts involved in these shifts are extremely small. The smallness of the deposit in the aortae—only 1/3000 of 1 percent of cholesterol administered—explains why the accumulation of deposits in the aortae takes as long as known from the course of human disease, and supports the view that cholesterol deposition is not the primary damaging factor in atherosclerosis.

Summary. Cholesterol-C^{14} and cholesterol-H^3 were fed to rabbits for 1 week and cholesterol was isolated from aortae, livers, small intestines, adrenals, kidneys, brains and blood. Isolated material from all tissues had a lower count than the material fed. It was surprising that cholesterol-H^3 counts were always comparatively lower than cholesterol-C^{14} counts. This discrepancy may be explained by a peripheral oxidation of the cholesterol-H^3 molecule, in which it loses radioactive hydrogen and therefore radioactivity. This peripheral oxidation is apparently mainly operative in blood. If after feeding of radioactive material non-radioactive cholesterol is fed for 7 or 11 weeks, radioactivity in both types of experiments is reduced to a small fraction of original values. From these data one can calculate that about 0.03 mg cholesterol are laid down in the aorta/animal/day/g of wet tissue, a very small part of the 900 mg fed/day/animal. The experiments show that there is a continuous exchange of radioactive cholesterol between tissues and blood in which even the plaques, formed in the aortae, must take part.

The help of Erszebet Joachim, Eva Moring and Deirdre McLeod is gratefully acknowledged.

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Propagation of Columbia SK Virus in Ehrlich Ascites Tumor Cells with Oncolysis. (25614)

Eiichi Furusawa and Windsor Cutting
Dept. of Medical Microbiology Stanford University, Calif.

Virus propagation in Ehrlich ascites tumor cells first reported by Koprowska and Koprowski(10) included the Mengo and EMC viruses of the Columbia SK group. The severe oncolysis was confirmed by Flanagan and Colter(3), and Levy and Snellbaker(11). Growth of these viruses and the F virus(8) and the MM virus(12) in cell culture also produced cell damage. Only Columbia SK virus of this group has failed to reproduce or to show cytopathogenic effects in tissue culture, though it may interfere with growth of other viruses(9). We now report the propagation of the SK virus in tumor cells with severe oncolysis.

Materials and methods. Ehrlich ascites cells were inoculated with Columbia SK virus from mouse brain passage. A mixture of 0.1 cc of 10^{-2} dilution of original brain suspension containing about 10^6 LD_{50} of virus and 0.3 cc of Ehrlich ascites fluid (cells) was inoculated into the peritoneal cavity of 3 to 5 mice. Before the mice died 3 to 5 days after inoculation, the infected ascites were aspirated, the cavity washed with 2 cc of saline, and 0.1 cc of ascites-saline fluid was inoculated, together with 0.3 cc of fresh Ehrlich cells, into new mice. This procedure was repeated through 4 generations. In fifth and subsequent generations the procedure was altered as follows: Infected ascites were centrifuged and 0.1 cc of 10^{-3} dilution of the supernatant containing no cells used as the next inoculum. Transmission was followed for 27 generations. Control mice were similarly inoculated with 0.1 cc of saline mixed with 0.3 cc of Ehrlich cells in place of the virus-Ehrlich cell mixture. Smears were prepared of each ascitic fluid, fixed with methanol, then stained with Giemsa solution and degree of cell damage observed. Some preparations were stained with acridin orange and studied in the fluorescent microscope(7). At each generation number of cells in the ascitic fluid was counted, and titer of virus and hemagglutinin calculated. For the hemagglutination and antihemagglutination tests, 1% of sheep red cells suspended in Mayer's barbital buffered saline was used(6). Anti-SK serum was made in rabbits by several injections of the source virus from mouse brain.

Results. 1. Oncolytic effect of Columbia SK virus on Ehrlich tumor cells. At the first inoculation with the virus from mouse brain, there was moderate cell degeneration, chiefly an increase of vacuoles in the tumor cells. This was much less pronounced in the second, third and fourth generations, but during these generations blind passages were continued. At the fifth generation the infected ascites became semi-transparent, with a decrease in cell count, and in smear preparations severe cell damage to most of the cancer cells was seen. The nuclei were pycnotic and karyorrhectic, the nucleoli had disappeared and the chromatin was rough. Many cells were of irregular shape, and in others the cell wall disrupted with freed nuclei. Many vacuoles were observed in the nuclei and cytoplasm of the cells considered infected. Cytoplasm of the damaged cells stained poorly and very little orange fluorescence, specific for ribonucleic acid, could be seen after staining with acridin orange. No specific findings such as inclusion bodies or cytoplasmic masses could be observed. From the 5th to the 27th (present) generation, except for the 10th, destruction of tumor cells mentioned continued in from moderate to severe degree (Table I). Cell counts made at the 9th and at a number of subsequent generations showed a marked diminution in number of cells (Table I). The mice were usually paralyzed by the virus infection by the 4th day and dead on the 4th to 5th day. Fig. 1 shows the typical cytopathogenic changes.

2. Hemagglutination of infected ascites. Agglutination of sheep red cells by ascitic

TABLE I. Oncolytic and Hemagglutination Effects of Columbia SK Virus on Ehrlich Tumor Cells.

	Oncolytic effects 3-4 days after infection		
No. of passage	Cell destruction	Total No. of cells in fluid	HA titer of cell-free ascitic fluid
Control	—	10^{7-8}	0
1	—		
2	—		
3	—		4
4	—		4
5	+		128
6	+	10^6	32
7	+	10^6	64
8	+		
9	+	3×10^6	
10	—	10^8	8
11	+	2×10^6	
12	+		32
13	+		16
14	+	5×10^5	16
15	+	10^6	256
16	+	4×10^6	
17	+	5×10^5	16
18	+		
19	+	10^5	256
20	+	5×10^5	
21	+	10^5	
22	+		
23	+	3×10^6	
24	+	3×10^6	
25	+	10^5	256
26	+	10^6	

fluid was titrated by the method of Horvath and Jungeblut(6); no hemagglutinin was detected with either the cell-free portion of normal ascitic fluid or the resuspended cancer cells. Hemagglutinins were detectable in varying titer in the infected ascitic cell-free fluid (Table I). Maximum titer was 1:256, noted in the 16th, 19th and 25th generations. Hemagglutinin titer in the tumor cells (when liberated by cell destruction using dry ice and acetone) was higher than in the cell-free fluid: 1:128 as compared with 1:32 at the 12th generation, and 1:32 as compared with 1:12 at the 13th generation.

3. *Virus titration of infected ascites.* Infected fluids diluted to 10^{-3} at the 5th, 8th and 9th generations were inoculated intraperitoneally without cancer cells, with survival of all the mice. However, when the inoculations were accompanied by uninfected cancer cells, all the mice became paralyzed within 5 days. The titer of active virus contained in cell-free fluid of the 7th generation was 10^5 LD_{50} per 0.1 ml when it had been inoculated with Ehrlich cells, but only 10^2 LD_{50} when inoculated intraperitoneally without cancer cells. This phenomenon was presumably due to adaptation of virus to the Ehrlich cells and to its more rapid propagation in them. When the virus was injected intraperitoneally and the cancer subcutaneously, or conversely, evidence of virus infection, *i.e.*, paralysis, was delayed for 3 to 5 days. Results with disrupted cells were the same as with the cell-free ascitic fluid. Amount of intracellular virus in both the 7th and the 15th generations was 10^5 LD_{50} per 0.1 ml after cell destruction using dry ice and acetone.

4. *Antihemagglutination tests.* The antigenicity of the adapted strain was tested with

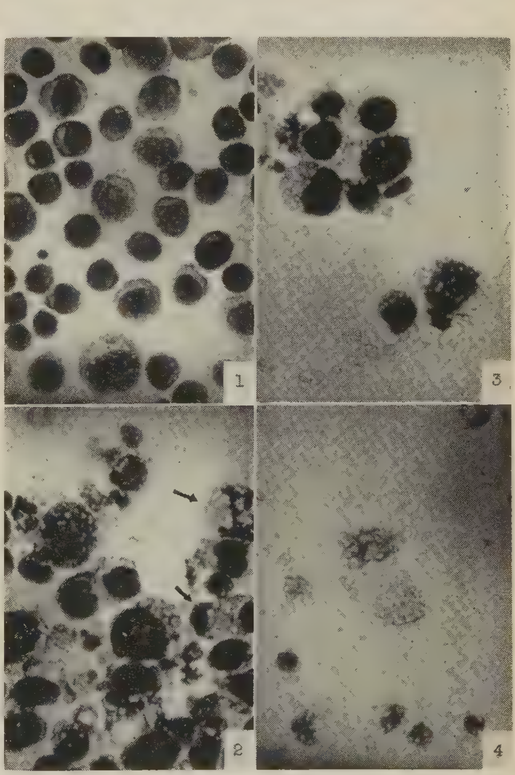

FIG. 1. Oncolytic changes produced in Ehrlich ascites cells by infection with adapted Columbia SK virus. 1. Normal Ehrlich ascites cells. 2. Infected cells (11th generation, 3 days after infection). Many vacuoles in most of the cells, and even in the mitotic cells (arrows). 3. Infected cells (27th generation, 3 days after infection) showing vacuolar degeneration. 4. Infected cells (27th generation, 4 days after infection) showing oncolysis.

TABLE II. Effect of Immune Serum on Oncolysis, Neutralization and Anti-Hemagglutination.

Serum	Oncolysis	Total cell count	Anti-HA titer†	Virus titer neutralized
Normal*	+	3×10^6	1:2	0
Immune*	—	3×10^7	1:640	10^{-3}‡

* Using 10^{-1} dilution of sera adsorbed with Ehrlich cells.

† Using 4 units of hemagglutinin of 19th infected ascites.

‡ 0.1 cc of 10^{-3} dilution of 19th passage ascites, containing about 10^2 LD_{50} of virus, was neutralized by 0.1 cc of 10^{-1} dilution of immune serum.

anti-SK rabbit serum made by the subcutaneous infections of mouse brain virus once a week for 4 weeks. In the last inoculum the virus was mixed with Freund adjuvant. The anti-HA titer of the serum from rabbits inoculated with the adapted strain was 1:64 after the third inoculation (preceding the Freund adjuvant); after the fourth inoculation it was 1:1280.

5. Neutralization tests. After adsorption (using the Ehrlich cells) of non-specific antibody against mouse tissues, a 10^{-1} dilution of the immune serum was mixed with a 10^{-3} dilution of infected fluid from the 19th generation plus Ehrlich cells. This mixture was then inoculated into mice. Control mice were injected with the same dilution of virus and Ehrlich cells in normal rabbit serum. After 3 days all control mice became paralyzed, and the cancer cells showed great destruction, while mice inoculated with the immune serum mixture were healthy and the cancer cells escaped oncolysis (Table II). Ehrlich cells mixed with the infected ascitic fluid (21st generation) were free of oncolysis when inoculated into the abdominal cavities of mice immunized by injection of ultraviolet-inactivated brain virus once a week for 4 weeks, showing similar antigenicity in the original brain virus and the adapted strain.

6. Trials against cell cultures of Sarcoma 180. To study the cytopathogenicity of the adapted SK virus, infected ascites from the 19th, 24th and 25th generations were inoculated into established cell cultures of Sarcoma 180 growing in Medium 199 with 5% of calf serum. No cell destruction occurred. The 19th inoculum was passed at 4-day intervals through 3 generations at 10^{-1} dilution, but no cytopathogenic effect appeared. Third generation inoculum was injected into mice together with Ehrlich cells, but none of the mice became paralyzed.

Discussion. The evidence that the virus was adapted to and reproduced in the tumor cells lies principally in the fact that the adapted virus alone had a low infectivity titer, but when given with Ehrlich tumor cells was highly lethal to the mice. Thus, adapted virus showed a titer of 10^5 LD_{50} per 0.1 ml when inoculated with tumor cells, but only 10^2 LD_{50} when inoculated without such cells. This thousand-fold difference may be interpreted as indicating that 99.9% of the virus was adapted to the Ehrlich cells and was incapable of producing death without passage through such cells.

Adaptation of other viruses to Ehrlich ascites cells has been accomplished or attempted: Influenza by Ackermann(1), NDV by Flanagan, Love and Tesar(4), Mengo by Flanagan and Colter(3), and vaccinia by Cassel(2) and by Furusawa, Toyoshima and Miyagawa(5).

Summary. Propagation of Columbia SK virus in Ehrlich ascites cells *in vivo* is described. After several passages an adapted strain of SK virus having an oncolytic effect was obtained. The adapted strain was relatively non-pathogenic for mice unless accompanied by Ehrlich cells, in which it could propagate freely. This strain, after many passages, maintains ability to agglutinate sheep red cells and is neutralized by antiserum against the original brain virus. The strain shows no cytopathogenicity to Sarcoma 180 cells in tissue culture.

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Potentiation of Chlorpromazine by Insulin.* (25615)

JACQUES A. LEBLANC
Dept. of Physiology, School of Med., Laval University, Quebec, Canada

Soon after its tranquilizing effect had been described(1) chlorpromazine was shown to decrease significantly the oxygen consumption of the whole animal(2). This effect combined with inhibition by chlorpromazine on the heat regulating center of the hypothalamus(1), and with the increased peripheral heat lost(3) constitute the main factors responsible for the marked hypothermia in animals injected with chlorpromazine. Other studies, which have shown that chlorpromazine inhibits activities of both cytochrome oxydase and adenosine triphosphatase(4,5,6) indicate that the decrease in metabolism caused by chlorpromazine may be due to some disturbances in metabolic pathways. Similarly insulin is also known to cause some inhibition in metabolic pathways as evidenced by its effect on hexokinase (7). It is also generally accepted that the depletion of substrate for cerebral metabolism, which is caused by insulin, is the sole explanation for the neural effect of this insulin on the brain and for its use in psychiatry(8). For these reasons it was postulated that chlorpromazine and insulin could potentiate one another on their effect on oxygen consumption or body temperature and on sleeping time of short-acting barbiturates such as hexobarbital.

Materials and methods. Two experiments were performed. In the first male albino rats, weighing 200 to 250 g, were used. The criterium for evaluating response to chlorpromazine was changes in rectal temperature as estimated by inserting a thermistor to a depth of 40 mm. Blood glucose determinations were also made on some groups of the first experiment. In *this experiment* 4 groups of 6 rats were used. Group 1 received chlorpromazine[†] (CPZ) (1 mg/kg); Group 2, CPZ (5 mg/kg); Group 3, CPZ (10 mg/kg); Group 4, CPZ (1 mg/kg) plus insulin regular (IR) (4 I.U./kg); Group 5 CPZ (5 mg/kg) plus IR (4 I.U./kg); and Group 6, CPZ (10 mg/kg) plus IR (4 I.U./kg). In the *second experiment* 4 groups of mice weighing 20 g were used. All 5 groups received hexobarbital (100 mg/kg). In addition Group 2 received IR (4 I.U./kg), Group 3 CPZ (1 mg/kg) and Group 4 regular insulin (4 I.U./kg) plus CPZ (1 mg/kg). All substances were injected intraperitoneally. Insulin and CPZ were injected, one hour and one half-hour respectively, before injection of hexobarbital. Sleeping time was the length of time between loss and reestablishment of righting reflex(9).

Results. A. *Potentiation of chlorpromazine hypothermia by insulin.* The results obtained in the first experiment are illustrated in Fig. 1 and 2. Fig. 1 shows that if the dose of chlorpromazine is sufficient to induce hypothermia (5 and 10 mg/kg), insulin potentiates this drop in body temperature. Furthermore, a dose of chlorpromazine of 5 mg/kg is potentiated sooner than a higher dose (Fig. 2); however insulin potentiates for a longer time a larger dose of chlorpromazine (10 mg/kg). In other words insulin by itself has no effect on body temperature but it greatly potentiates the hypothermia of chlorpromazine. Blood sugar determinations indicate that

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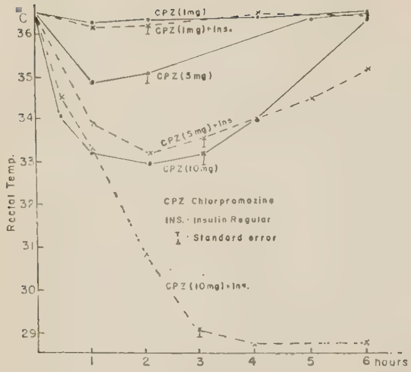
[†] Chlorpromazine was generously supplied by Poulenc Ltd. under trade name of Largactil.



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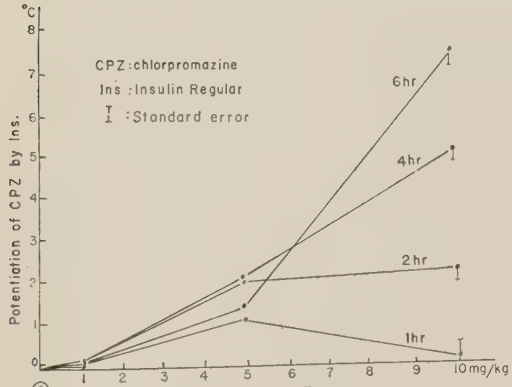

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FIG. 1. Potentiation of chlorpromazine hypothermia by insulin (4 I.U./kg).

FIG. 2. Effect of insulin (4 I.U./kg) on hypothermia induced by different doses of chlorpromazine.

chlorpromazine (10 mg/kg) increases blood sugar (132 ± 8 mg %) as compare to the normal value of 98 ± 6 mg %. However simultaneous injection of chlorpromazine (10 mg/kg) plus insulin (4 I.R./kg) produces a marked hypoglycemia (52 ± 4 mg %) which resembles that of insulin when used alone (49 ± 5 mg %).

TABLE I. Increase by Chlorpromazine of Hexobarbital Sleeping Time and Potentiation of This Increase by Insulin.

Treatment	Sleeping time (min.)	No. of animals	Value of "p"
Hexobarbital (100 mg/kg)	30 ± 3	9	>.01
Idem + chlorpromazine (1 mg/kg)	51 ± 5	8	.05
Hexobarbital (100 mg/kg) + insulin (4 I.U./kg)	36 ± 4	9	>.01
Idem + chlorpromazine (1 mg/kg)	82 ± 9	7	

B. *Potentiation of chlorpromazine increase in hexobarbital sleeping time by insulin.* The results are summarized in Table I. Insulin has no effect on hexobarbital sleeping time. However when used with chlorpromazine it greatly potentiates the effect of this substance. The increased sleeping time induced by chlorpromazine in animals receiving hexobarbital is larger still if the animals are injected simultaneously with insulin.

Discussion. Insulin, at the levels used in our study, has no effect on body temperature of rats or on sleeping time of hexobarbital in mice. However when used with chlorpromazine it exaggerates the hypothermia and prolonged hexobarbital sleeping time normally observed with this tranquilizer. Consequently a marked potentiation of chlorpromazine by insulin was obtained. However chlorpromazine has no effect on insulin hypoglycemia, since our results show that the marked hypoglycemia of insulin is not changed by chlorpromazine. This result confirms previous reports(10,11) that chlorpromazine does not alter the hypoglycemia of insulin.

The 2 criteria used in this study are hypothermia and hexobarbital sleeping time. Since these two functions are centrally controlled and both exaggerated by chlorpromazine, it is possible that potentiation of chlorpromazine by insulin originates in the central nervous system.

Summary. The action of insulin as potentiator of chlorpromazine was tested. Under conditions of our experiment, insulin had no significant effect on body temperature of rats and on sleeping time of hexobarbital in mice. However insulin greatly potentiates the hypothermia and prolonged hexobarbital sleeping time normally caused by chlorpromazine. On the other hand the hypoglycemic effect of insulin was not potentiated by chlorpromazine.

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Cytopathogenicity and Plaque Formation with Lymphocytic Choriomeningitis Virus.* (25616)

Lois M. Benson and John E. Hotchin (Introduced by Rachel Brown)
Division of Laboratories and Research, N. Y. State Dept. of Health, Albany

During experiments(1) aimed at a better understanding of pathogenesis of lymphocytic choriomeningitis (LCM) virus infection in mice and ability of infant mice to develop immunological tolerance to it, an attempt was made to obtain cytopathic effects (CPE) and a plaque assay with this virus. Previous work indicated that LCM virus is not readily cytopathogenic in tissue culture, except in KB cells(2). An earlier attempt to obtain a plaque assay with these cells was not successful. In our hands several other cell lines including HeLa, Detroit 6, human intestine, human liver, human kidney, FL, primary human amnion and L cells have shown no effects in spite of virus multiplication. However chick embryo tissue cultures appeared to have possibilities since infected cultures, kept 3 weeks or longer, showed decreased acid production with less luxuriant growth than controls. Experiments were therefore set up in which LCM mouse brain virus (the WE strain obtained originally from Rockefeller Inst.)† was passed in series through chick embryo tissue cultures.

Methods. Cells were prepared from 9-day chick embryos using Dulbecco's method(3). One ml of tissue suspension at a concentration of 300,000 cells/ml in growth medium was used to seed 16 × 125 mm screw-capped test tubes. The nutrient medium consisted of 20% calf serum, 5% chick embryo extract, 75% Medium 199 with penicillin and streptomycin. After 2 days' incubation, cultures were washed with Hanks tris pH 7.2 (M/50 tri (hydroxymethyl) amino methane in Hanks saline) and infected with LCM virus. A cytopathic effect was produced in the first chick embryo tube passage. In 6 days LCM infected cultures appeared slightly more granular than controls and the sheets were beginning to break. In 9 days the sheets in infected cultures were completely broken up, only single cells remaining, whereas cell sheets in control tube were intact. At this time pH of control culture fluid was 6.8 and pH of LCM culture fluid was 7.5 or higher. Forty serial passages of this virus have been made using at first 3- or 4-day and then 7-day fluid from LCM infected cultures as inoculum; the final passes produced a moderate CPE in 5 days and a moderate color change in 7 days. Control culture fluid has been passaged similarly, and has shown no CPE.

Results. Mice inoculated intracerebrally with LCM culture fluids at various passage levels, diluted 10^{-3} or 10^{-4}, produced typical LCM convulsions and deaths. Undiluted control fluids never caused illness or death of mice. Titrations indicated that maximum virus titer in tubes was reached by 7th day. CPE was obtained in terminal dilution (10^{-4}) tubes after 12 days; no new CPE appeared later. In a neutralization test in mice, virus

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† Kindly supplied by Dr. F. P. Nagler, Dept. of Nat. Health and Welfare, Ottawa, Canada.

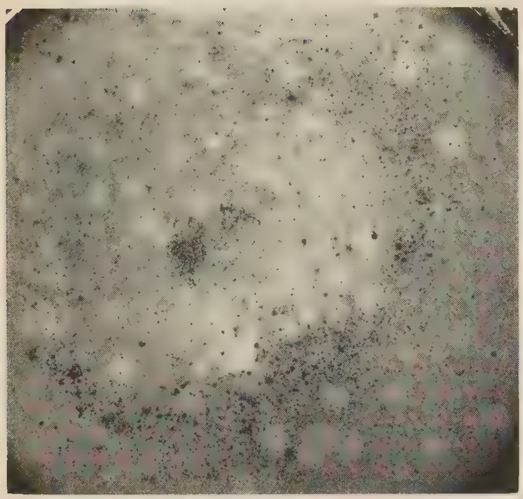

FIG. 1. Photograph of 10 cm Petri plate showing LCM plaques 13 days after inoculation of chick cell monolayer.

in 7-day fluid was neutralized at 10^{-2} but not 10^{-1} by anti-LCM guinea pig serum, but not by normal guinea pig serum. The 7-day fluid gave a positive result by complement-fixation test without anticomplementary effects with specific antiserum.

An attempt was made to produce plaques on chick embryo monolayers with the chick cell adapted strain. Monolayers were made by seeding 10 ml of cells at concentrations of 1.2-1.5 × 10^6/ml in 10 cm Petri plates. Monolayers were inoculated with 0.5 ml of inoculum and incubated for ¾ hour adsorption period. Overlay (9 ml/plate) was composed of 15% tryptose phosphate broth and 1% agar in M 199. On 2nd, 6th, and 10th days, additional overlays (3 ml) were added, the last one containing 1/8000 neutral red. Round plaques approximately 5 mm in diameter appeared on 12th day (Fig. 1). On 4 occasions 3 plaques and 3 adjacent "non-plaque" areas were removed, each emulsified in 1 ml of 10% normal guinea pig serum in saline and inoculated intracerebrally (0.03 ml) into 5 mice. All mice inoculated with undiluted plaque suspensions died with typical LCM convulsions, whereas mice receiving control area extracts remained healthy.

A surprising result was obtained when monolayers were infected with 20% LCM infected mouse brain suspension (original virus unadapted to tissue culture) and treated as above. Plaques appeared on 12th day and were apparently the same as those produced with tissue culture adapted virus. A comparison was made /ml of virus preparation of sensitivities of the following methods of assay: plaque assay expressed as plaque-forming units (PFU), tissue culture assay expressed as 50% infective dose ($TCID_{50}$) as determined by cytopathogenicity, and 50% mouse lethal dose assay (LD_{50}) by intracerebral inoculation. Five different preparations were assayed by both PFU and LD_{50}, and 7 by both $TCID_{50}$ and LD_{50} (Tables I, II). Average ratio LD_{50}/ml: $TCID_{50}$/ml: PFU/ml was 1:0.73:0.23, indicating a very close agreement between the 3 methods, with descending sensitivity between mouse, tissue culture, and plaque methods.

A disadvantage of this assay has been the difficulty experienced in regularly keeping the monolayers alive for a minimum of 12 days. Duration of cell viability varied between a maximum of 20 and a minimum of 10 days.

Summary and conclusions. At first it was thought that 40 passages of the virus in chick tissue had increased its cytopathogenicity to the point where plaque production could oc-

TABLE I. Relationship Between Plaque Titer (PFU/ml) and Mouse Titer (I.C. Inoculation, LD_{50}/ml) of LCM Virus Preparations.

Avg No. of PFU/ml	Mouse titer expressed in LD_{50}/ml
1.6 × 10^4	3.3 × 10^4
3.7 × 10^3	1.65 × ”
1.6 × 10^4	1.04 × 10^5
2.9 × 10^3	*Idem*
2.6 × 10^4	”

TABLE II. Relationship Between Mouse Titer (LD_{50}) and Tissue Culture Titer ($TCID_{50}$) of LCM Virus Preparations.

No. of days harvested	Mouse titer expressed as LD_{50}/ml	Titer expressed as $TCID_{50}$/ml
1	3.3 × 10^4	7.2 × 10^3
2	5.2 × 10^3	1. × 10^4
5	1.65 × 10^4	1. × 10^5
6	*Idem*	*Idem*
7	5.2 × 10^5	7.2 × 10^4
8	1.65 × ”	*Idem*
9	5.2 × ”	1. × 10^5

cur. However ability of non-adapted strain to produce plaques refutes this conclusion. In retrospect it appears that no actual change in virus had occurred during serial passage, but the observation that CPE could be regularly produced after as long as 12 days led to use of above procedure and successful production of plaques appearing with this virus at relatively late time of 12 days after inoculation. The sudden appearance of plaques seems unusual; apparently the final change of CPE, resulting in loss of neutral red staining, must occur very suddenly with this system, resulting in overnight appearance of plaques.

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Agar Diffusion Method for Detection and Bioassay of Antiviral Antibiotics.
(25617)

ERNEST C. HERRMANN, JR., JANIS GABLIKS, CLAIRE ENGLE AND PRESTON L. PERLMAN
Biochemistry Dept., Schering Corp., Bloomfield, N. J.

The discovery of antibacterial and antifungal antibiotics clearly indicates that microbial cultures contain a wealth of diverse biochemicals useful in infectious disease. Unfortunately, there has been little progress in the exploitation of such microbial products for treatment of human and animal viral diseases. A major reason for this failure may lie in the time-consuming, cumbersome and insensitive technics that until recently were a necessary feature of virus research. Such technics did not permit large-scale screening of microbial cultures important to the discovery of clinically useful antibiotics. Furthermore, even if a naturally occurring antiviral agent were discovered, there were no satisfactory bioassay systems available to cope with the hundreds of assays essential to successful fermentation and isolation of the active principle. The following is a description of a filter paper disc-agar plate technic, similar to that used in bacteriology as applied to screening and assay of antiviral agents. The suggestion that such application was possible was made by De Somer and Prinzie(1) in their description of a similar method used for measurement of poliovirus antibody. More recently, Porterfield (2) applied this approach to interferon bioassay.

Materials and methods. Preparation of virus-infected agar plates. Trypsinized chick embryo cells were prepared as described by Dulbecco(3), suspended in Melnick's lactalbumin hydrolysate-calf serum medium(4) with Earle's bicarbonate balanced salt solution, to produce 3.5×10^6 cells per ml. The cell suspension was adjusted to pH 7 with CO_2, then poured into acid dichromate cleaned, sterile Pyrex baking dishes (10 × 6½ inches), 85 ml per dish. The dishes were sealed air tight with Saran Wrap (Dow Chemical Corp.) and the medium readjusted with CO_2 to a pH 7.5 if required. This second gassing is often necessary because of the rapid loss of CO_2 from the relatively high bicarbonate containing medium during incubation period. About 5 to 10 chick embryos, depending on their age, were required to produce enough cells for a single dish. Eleven-day-old embryos seemed to produce the best cell yield. After 48 hr incubation at 36°C the chick embryo cells grew into a confluent sheet; the medium was discarded and the cell culture infected with a 5 ml suspension of one of the following viruses: West Nile virus (Egypt 101 strain from Dr. Joseph Melnick, Baylor Univ.), vaccinia (WR, mouse neurotropic), herpes simplex (HF378) and Newcastle disease (California No. 11914) viruses obtained from American Type Culture Collection. The virus was incubated with the cells for 1 hr, then 65 ml of overlay medium con-

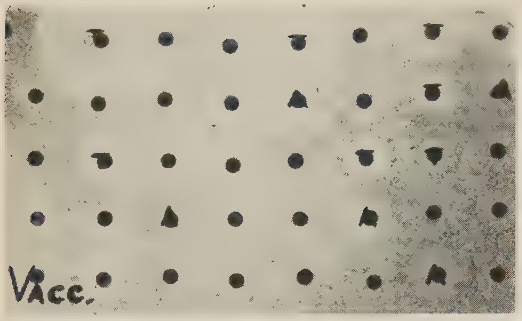

FIG. 1. Composite screening plate using vaccinia virus. Test discs intentionally used to show appearance of zones produced by a toxic substance (T) and zones resulting from an antiviral material (A).

taining 1.5% Noble's agar (Difco) was poured over the cell sheet. This medium contains the same constituents as Melnick's medium with the omission of phenol red and replacement of the 2% calf serum with Difco yeast extract (0.1% final concentration). The plates were again sealed with fresh Saran Wrap and further incubated 3 to 4 days, depending on the virus used, to permit formation of plaques. At the end of this period 36 ml of melted 1.5% agar containing 90 mg/100 ml of indonitro-tetrazolium chloride (Dajac) was poured over the primary agar base. Within 2 to 4 hr at 36°C the living cells reduced the tetrazolium salt to a purple-red formazan. Dead cells and hence virus-induced plaques were colorless. This staining technic, although expensive, has advantages over neutral red which also can be used. With neutral red the color is lost as soon as the cell dies while tetrazolium salt produces a permanent stain not affected by the later death of the cell. Further, light has no apparent deleterious effect on the tetrazolium system as has been observed with neutral red(5). *Preparation and application of filter paper discs.* A variety of number and letter designations, representing various test materials, were typewritten on strips of filter paper (Schleicher and Schuell, type 470), then punched out with a standard ¼-inch paper punch. The resulting ¼ inch discs were touched to the surfaces of either whole microbial liquid cultures or solutions of synthetic compounds. The discs were dried, sterilized with ethylene oxide, and placed on the hardened surface of the primary agar layer, remaining in place during the entire period of plaque development (3-4 days). The discs were later covered by the second, stain-containing agar overlay.

Results. Screening. Between 25 and 40 filter paper discs can be tested in a single baking dish (Fig. 1) making it possible to test large numbers of substances with relative ease. When a disc contained a substance inhibiting plaque formation, a distinct halo of deeply stained, living cells free of plaques (Fig. 2) was produced. The same photograph shows the disc containing the active principle slightly displaced so an inner, small zone can also be seen. This latter zone resulted from the presence of a cytotoxic agent, illustrating how toxicity of a test material can be easily distinguished from virus cytopathogenicity. These differences could not be readily determined if liquid medium tissue culture systems had been used. Care was taken to use enough virus to produce numerous plaques but not so much as to destroy the entire cell sheet. In this way the general condition of the cell sheet could be determined and toxic test materials detected. Plaque-free zones up to 40 mm in diameter were routinely produced by discs impregnated with unconcentrated, liquid bacterial cultures that contained inhibitors of plaque formation.

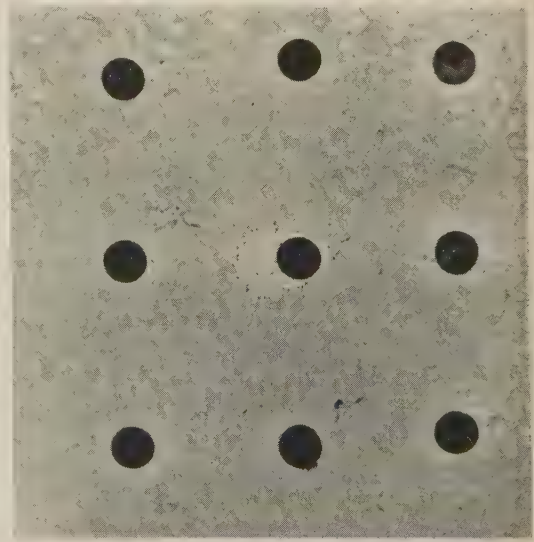

FIG. 2. Vaccinia virus plaque-free zone produced by a disc impregnated with a bacterial culture broth containing an antiviral substance.

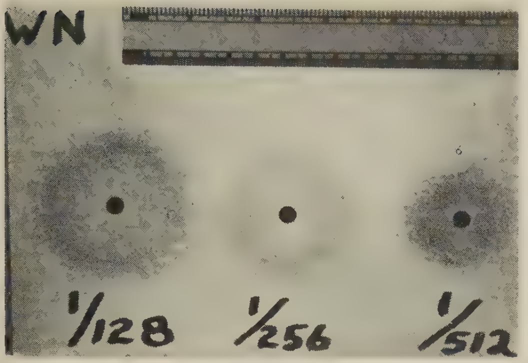

FIG. 3. Representative assay of an antiviral bacterial culture. Discs impregnated with indicated broth dilutions. In this example 10 times more West Nile virus was used than would be used in a routine screening test.

Bioassays were performed by impregnating paper discs with various 2-fold dilutions of an antiviral agent and placing the discs on the surface of a freshly prepared, virus infected, agar overlay culture. The size of the plaque-free zones produced were in proportion to quantity of active material present in the disc (Fig. 3). So far, it has not been possible to produce zones with regular, well-defined edges, even though a variety of virus concentrations have been used. Perhaps as much as a 10% error resulted from this inability to define accurately the zone sizes. Nevertheless, adequate bioassays were done. As the data show (Fig. 4), when zone diameter was plotted against dilution used, similar slopes resulted for a single antiviral agent no matter which virus was used. It was found best, however, to use discs containing enough active material to produce zones in the range of 20 to 50 mm for the most reproducible data. There was also some indication that concentrations of semipurified antiviral agents at less than 1 μg per disc produce zones in that range.

All the problems inherent in any agar diffusion method(6) are obviously present in this system. In addition, however, there are other problems unique to a tissue culture method. For example, it was not possible to use wide variations in pH in an effort to enhance diffusion of an antibiotic since the tissue culture cells were very sensitive to a non-physiological pH. Further, the cells were considerably more sensitive to non-specific toxic agents than bacteria would be in a similar test.

In the experiments reported here, the Pyrex baking dishes did not have perfectly even bottoms hence the depth of the agar varied, often resulting in asymmetric zone shapes. Despite these and other problems, the data produced readily answered various questions associated with fermentation and isolation of an antiviral microbial product.

It is, of course, usually only necessary to test one concentration of the agent in question once a standard has been established. At this point assays of any one material can be performed using a single disc and comparing the zone produced to that produced by a disc containing a standard quantity of the same substance. This permits bioassay of a very large number of samples with a minimum of effort.

Discussion. It now appears that the technics used so successfully in the search for and development of antibacterial antibiotics can be applied directly to the problems of virology. Not only can a very large number of materials be tested but the critical requirement for a bioassay can be fulfilled. Further, preliminary work already indicates that the

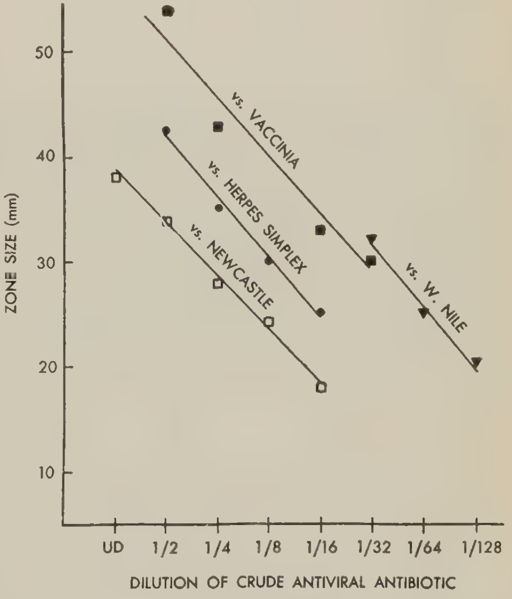

FIG. 4. Representative assay results of a single antiviral antibiotic tested against 4 different viruses.

bioautograph technic of developing paper chromatograms of antibacterial agents can also be applied to antiviral materials. It remains to be seen, however, whether antiviral substances discovered by this technic will eventually be useful in human and veterinary medicine. It is encouraging, in this regard, that at least one antiviral agent—isatin-thiosemicarbazone—which is highly active in vaccinia virus infected mice(7), also was extremely active in this tissue culture system. It can only be hoped that the success observed in discovering clinically useful antibacterial agents can be paralleled in virology.

Summary. A procedure has been developed whereby agar diffusion technics used in bacteriology can be applied to antiviral agents. Chick embryo cell monolayers were grown in Pyrex baking dishes, infected with virus and overlaid with agar. Filter paper discs impregnated with the test materials were deposited on the hardened agar surface. After proper incubation the virus produced plaques in the cell sheet, except in the area around a disc containing an antiviral agent. Since size of the plaque-free zone was proportioned to concentration of the antiviral agent present in the disc, bioassays were simply performed in exactly the same manner as done with antibacterial antibiotics.

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Activation of Histamine-Releasing Factor in Normal Rat Plasma by *E. coli* Endotoxin. (25618)

SHELDON E. GREISMAN
Depts. of Medicine and Physiology, University of Maryland School of Medicine, Baltimore

The mechanisms whereby Gram-negative bacterial endotoxins (somatic antigens) produce the various pathophysiologic alterations in the mammalian host(1) are poorly defined. The basic problem of whether endotoxins act directly at the cellular level, or through an intermediate step whereby composition of blood is altered, or both, is unresolved. The toxic activity of bacterial endotoxins, which are protein-lipid-polysaccharide complexes, appears to reside in the lipid-polysaccharide moiety(2). In a previous study(3), polysaccharides prepared from somatic antigens of *Salmonella typhi* and *Salmonella paratyphi* were included among a list of numerous high molecular weight polysaccharides capable of activating, *in vitro,* a factor in normal rat plasma with potent histamine-liberating activity. It seemed possible, therefore, that certain of the pathologic effects induced by bacterial endotoxin might be attributable to activation of such plasma histamine-liberating factors. The previous study, however, utilized large quantities of the bacterial polysaccharides (20 mg/ml rat plasma) and failed to specify whether these a) were combined with or dissociated from their lipid or protein components, b) exhibited the characteristic activities of endotoxin *in vivo,* or c) released some portion of histamine by direct tissue action. The present study was designed to determine whether smaller quantities of physiologically active, purified lipopolysaccharides from Gram-negative bacteria could activate plasma histamine-releasing factors and to exclude the possibility that such histamine release might be attributable to direct endotoxin-tissue interaction.

Materials and methods. The lower ileum was resected from normal 250 g guinea pigs

TABLE I. Lethality of Single Intravenous *E. coli* Endotoxin Injections for Immature Albino Rabits.*

E. coli endotoxin† μg/kg	96 hr mortality (deaths/total)
200	0/30
500	1/20
1000	1/10
2000	0/5
4000	4/8
5000	2/6
7000	3/6
10,000	7/8

* Rabbit wt = 1.1 (range 0.9-1.5) kg.

† Supplied by Difco Laboratories as Lipopolysaccharide *E. coli* 0127B8.

after head blow sacrifice and placed in Tyrode solution at room temperature. Four cm segments were removed, attached to a lever arm exerting 5 g tension, and suspended in a 25 ml aerated 37°C Tyrode bath stirred by a glass rod connected to a constant speed motor. Multiple segments of ileum from each of 8 guinea pigs were studied as follows: Baseline responses to a standard histamine solution (0.1 μg histamine base in 0.1 ml physiologic saline) were recorded kymographically by adding histamine immediately after interrupting the aerating system. Following each contraction, the ileum was washed 3 times with Tyrode solution and aeration restarted. When reproducible histamine responses were obtained, the effect of 0.5 ml of the following solutions was tested in the sequence indicated; a) physiologic saline containing 5, 50, or 250 μg *E. coli* endotoxin, b) normal heparinized fresh rat plasma (100 μg heparin/ml plasma) diluted 1:1 with physiologic saline, and c) aliquots of the same rat plasma diluted 1:1 with physiologic saline, containing a final content of 5, 50, or 250 μg *E. coli* endotoxin. All endotoxin and control solutions were incubated at 37°C for 30 minutes prior to guinea pig ileum assay. With each solution tested, the Tyrode bath aeration was temporarily discontinued to circumvent formation of noxious aerosols. After each assay, the ileum was washed 5 times with Tyrode solution and reactivity to exogenous histamine rechecked. Ability of an antihistaminic compound, pyribenzamine (Ciba) to alter the ileum responses was also determined. The *E. coli* endotoxin employed for the present study was supplied by Difco Laboratories as *E. coli* lipopolysaccharide 0127B8 and prepared by a modification of the Boivin trichloracetic acid extraction procedure which removes most of the protein components(4). The lethality of this endotoxin preparation following single intravenous injections into immature healthy albino rabbits is given in Table I. In addition, 1 to 10 μg of the endotoxin injected intravenously generally produced delayed hemorrhagic necrosis in areas of the rabbit abdominal wall simultaneously treated with 100 μg epinephrine intradermally; 100 μg of the endotoxin injected intravenously provoked the local Shwartzman reaction in most skin areas prepared 24 hours previously with 50 μg endotoxin.

Results. Ten segments of guinea pig ileum were tested with the 250 μg aliquots of *E. coli* endotoxin; Fig. 1 is representative of results. The *E. coli* endotoxin *per se,* incubated at 37°C for 30 minutes in physiologic saline, failed to evoke contractile response during ileum contact. Following endotoxin washout, the ileum remained fully responsive to exogenous histamine. Normal fresh rat plasma, incubated with physiologic saline as described above, elicited minimal or no ileum responses with the dosages employed and left the ileum fully responsive to exogenous histamine. In contrast, mixtures of the rat plasma incubated with 250 μg *E. coli* endotoxin in physiologic

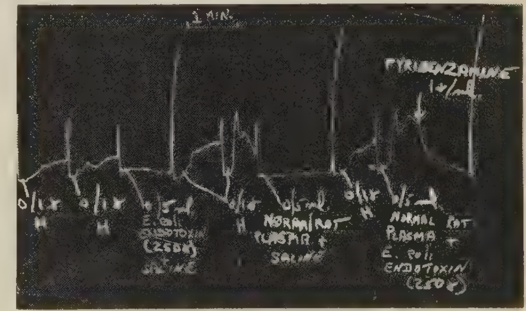

FIG. 1. Effect of a physiologically active lipopolysaccharide component of *E. coli* endotoxin on ability of fresh normal heparinized rat plasma to contract isolated guinea pig ileum segments at 37°C in a 25 ml Tyrode bath. (0/1γ H = 0.1 μg histamine base. The unique effect of 0.1 μg exogenous histamine in the 5th assay from the left was intentionally induced by introducing the histamine adjacent to the ileum for comparison with response to activated rat plasma. The pyribenzamine added, 1 μg/ml, is expressed as final concentration in the Tyrode bath.)

saline induced immediate ileum contraction which could be reversed rapidly by pyribenzamine in final concentrations of 1 μg/ml. Normal fresh rat plasma from 3 different pools was employed and each yielded comparable results.

The 50 μg aliquots of *E. coli* endotoxin were assayed on 12 fresh guinea pig ileum segments and the 5 μg aliquots on 8 other segments. A sequence of events entirely similar to that described above was obtained consistently with 3 different batches of fresh rat plasma. Although the assay system could not be employed for reliable quantitative data, when responsiveness of each ileum segment to the standard histamine solution was considered, it appeared that greater activation of the rat plasma "ileum contractor" factor could be accomplished with the 50 and 250 μg *E. coli* endotoxin than with the 5 μg aliquots.

In the above experiments, the *E. coli* endotoxin incubated in saline failed to induce contractions upon contact with guinea pig ileum segments during periods of 2 minutes or less. Although this was sufficient to indicate clearly the different response from that of endotoxin incubated in rat plasma, it seemed of interest also to determine whether endotoxin *per se,* if permitted longer tissue contact time or employed in large quantities, could directly induce ileum contraction. In addition, the previous experiments were performed without precautions to circumvent Tyrode solution or glassware contamination with traces of bacterial endotoxin. Conceivably, suspension of guinea pig ileum segments in Tyrode solution already containing endotoxin may render such segments refractory to endotoxin assay. Studies were therefore performed with pyrogen-free solutions and glassware rinsed with pyrogen-free saline and heated at 180°C for 2 hr. Each ileum segment was resected with strict aseptic technic and the ends firmly ligated to prevent fecal leakage. In 3 trials, 2000 μg aliquots of *E. coli* endotoxin were thus assayed, employing fresh guinea pig ileum segments from 2 pigs. No contractions or change in baseline tone resulted during contact periods of 20 minutes in each case. Similar results occurred in 3 other trials without pyrogen-free precautions. The baseline responsiveness of these ileum segments to standard exogenous histamine was ± 200% that shown in Fig. 1 and histamine responsiveness at completion of each assay remained essentially unaltered.

Discussion. The findings suggest that the lipopolysaccharide *E. coli* endotoxin preparation employed, exhibiting lethal, epinephrine-sensitizing, and Shwartzman provoking activity in the rabbit, was capable of activating a potent histamine (or histamine-like) liberating factor in fresh heparinized rat plasma upon 37°C incubation *in vitro.* The possibility that the pristine endotoxin exerted a direct tissue effect in the test system was excluded, although the alternative that rat plasma converted the endotoxin to a direct histamine-releasing material was not ruled out. The latter, however, appears improbable in view of previous studies on the nature of plasma histamine-liberating factor activation by polysaccharides(3). It is of interest that whereas 5 μg *E. coli* endotoxin incubated in rat plasma induced immediate and definite histamine liberation, 2000 μg of the same endotoxin in physiologic saline failed to induce detectable histamine release despite 20 minutes of tissue contact. This inability of *E. coli* endotoxin to release histamine directly did not appear to result from use of an assay system already contaminated with endotoxin since precautions to eliminate such contamination failed to alter the results. Nor could the failure to detect direct histamine release by *E. coli* endotoxin be attributed to a toxic depression of the histamine sensitivity of the assay system.

Interpretation of the present observations in relation to physiologic activity of endotoxin is speculative and must be approached with caution. First, it is possible that some contaminant, rather than *E. coli* endotoxin *per se,* was responsible for the rat plasma histamine-liberating factor activation; this seems unlikely, however, since polysaccharides from somatic antigens of *Salm. typhi* and *Salm. paratyphi* also activate such factors(3). Secondly, species specificity for activation of plasma histamine-releasing agents is unknown, although if endotoxin reacts as do other polysaccharides, fresh rat plasma would constitute the major suitable substrate(3). Finally, whether plasma activation can occur *in vivo*, and the plasma endotoxin concentrations required, remains unanswered. Since endotoxins can be cleared rapidly from the circulation(5), it is speculative whether endotoxin would remain in contact with plasma in concentrations and for periods sufficient to initiate histamine-releasing activation *in vivo;* presumably dosage, route of administration, and status of the reticulo-endothelial system would affect this factor(6). Nevertheless, if the rat behaves similarly to the mouse, *E. coli* endotoxin plasma levels after single intravenous dosages above LD_{50} may remain comparable to the present *in vitro* test levels for at least 3 hours(6). Moreover, considerably lower plasma concentrations of endotoxin might be effective *in vivo* since the *in vitro* treated rat plasma exhibited histamine-releasing activity after 100-fold dilution in the Tyrode bath.

Despite the most liberal assumptions, the ability of *E. coli* endotoxin to activate plasma histamine-releasing factors *in vitro* fails to account for many of the *in vivo* physiologic activities. Thus numerous other polysaccharides lacking classical endotoxic activity possess the same or greater *in vitro* plasma activating potential(3). Indeed, recent studies indicate that the lipid moiety of bacterial endotoxin is the toxic factor, and that the polysaccharide (which may constitute the plasma histamine-releasor activating factor) is important merely as a carrier to permit dispersion of the toxic lipid(7). The significance of the present observations therefore centers on the possibility that in some animal species, the plasma histamine-releasing mechanism might, by *contributing* to or *modifying* other concomitant reactions *in vivo,* participate in certain pathophysiologic alterations which characterize the endotoxic response. Some support is already available from evidence of Kuida *et al.*(8) that *E. coli* endotoxin action, at least on the vascular bed of the dog and cat lung, is not direct but mediated through liberation of some agent or agents in blood and that such intermediary substances have generalized smooth muscle constrictor activity. It was noted, however, that *exogenous* histamine in this system did not reproduce the entire endotoxin action. Additional evidence for a histamine-releasing agent is suggested by observations of Gilbert(9) that the initial acute circulatory responses of the cat to *E. coli* endotoxin are compatible with histamine and serotonin release; this parallels studies by Weil and Spink (10) in which histamine-like substances were detected in inferior vena caval blood of dogs within one to 2 minutes after *E. coli* injection. Finally, the striking clinical similarity between anaphylactic and endotoxin induced shock(10) is consistent with endotoxin activation of plasma histamine-liberating agents since such agents are believed capable of reproducing certain physiologic alterations of specific anaphylaxis(11,12). Future studies will be of interest to determine whether *in vitro* and *in vivo* histamine-liberating activity of *E. coli* endotoxin can be induced by the toxic lipid component described by Westphal (7) in the absence of the polysaccharide moiety.

Summary. Employing isolated guinea pig ileum segments in aerated Tyrode solution, 5 to 250 μg aliquots of a physiologically active lipopolysaccharide component of *E. coli* endotoxin were found capable of activating a potent histamine-like releasing factor upon *in vitro* incubation with 0.5 ml diluted fresh heparinized normal rat plasma. The release of histamine by this rat plasma factor occurred within seconds after tissue contact. The *E. coli* endotoxin preparation *per se,* in the dose-time relations employed, did not liberate histamine directly from the ileum segments; indeed, 2000 μg *E. coli* endotoxin failed to release detectable histamine during 20 minute tissue contact periods. This lack of histamine-releasing activity could not be attributed to preexisting endotoxin contamination of the tissue bath nor to toxic depression of the histamine sensitivity of the assay system. The above findings, in conjunction with previous evidence, suggest that *E. coli* lipopolysaccharide behaves, at least in one manner, as do numerous other high molecular weight polysaccharides and justifies the hypothesis that activation of plasma histamine-releasing factors may account for certain of the pathophysiologic alterations which characterize the endotoxic response. Further studies

are required to define the importance of the polysaccharide moiety of the endotoxin molecule for the histamine-releasing activity.

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Bactericidal Activity of Normal Serum Against Bacterial Cultures. II. Activity Against *Escherichia coli* Strains. (25619)

LOUIS H. MUSCHEL (Introduced by G. Edsall)

Division of Immunology, Walter Reed Army Inst. of Research, Washington, D.C.

The preceding paper of this series(1) on bactericidal action of normal serum against strains of *Salmonella typhosa* established that a strain's resistance to serum bactericidal action was associated with its O-inagglutinability or Vi content(2). The most resistant of the strains tested were a strain of maximum mouse virulence and of proven virulence for chimpanzees as well as another strain isolated during a severe human outbreak. From the point of view of the host, moreover, it was shown that natural bactericidal antibody of marked specificity played the major role in serum bactericidal action. The present report represents a study of bactericidal action of normal human serum against strains of *Escherichia coli.* Though usually non-pathogenic, this species was chosen for study because it may cause acute or chronic disease, and recognition of this disease is increasing. In addition, there is evidence that certain serological types of *E. coli* are responsible for acute enteritis in newborn infants and calves(3). For a given host, therefore, wide variations in virulence of different strains of the same species may exist. Distinguishing characteristics have been ascribed to potentially virulent strains of *E. coli.* These have included necrosis of the skin of rabbits and of cells in culture, hemolysis of horse erythrocytes, ability to elicit the Shwartzman phenomenon, greater mouse virulence, and O-inagglutinability(4,5). These properties have not led to well-defined distinctions between virulent and non-virulent strains. To these may now be added resistance of strains to bactericidal action of normal human serum. It would seem likely, therefore, that virulence results from an association of various properties, rather than from a single characteristic.

Materials and methods. E. coli strains. Most of the cultures and the information concerning their source were received through the generosity of Dr. W. H. Ewing, Enteric Bacteriology Unit, Communicable Disease Center, U.S.P.H.S., Chamblee, Ga., except for 0114 serotypes which were kindly provided by Dr. Joan Taylor, Central Public Health Lab., London. Most of the cultures were isolates from normal healthy children or from infants with diarrhea. *Sera.* Several pools of sera from normal humans were tested. Each pool was composed of equal volumes of sera of 4 individuals whose sera lacked detectable *E. coli* agglutinins against the test organisms. Tests with the different pools invariably gave

TABLE I. Bactericidal Action of Pooled Normal Human Serum Against *E. coli* Isolates from Normal Individuals.

A. Isolates from normal children

Serotype	ED_{50}, ml serum
02	.40
02	.35
03	.35
07	.34
07	.025
07	.017
021	.12
036	.14
037	.24
04	.17
0127a, 0127b:B10	.014
086a, 086b:B9	.012

B. 3 Isolates from one individual

Type	ED_{50}, ml serum
Anaerogenic 011	>.45
01	.028
091	.24

C. Isolates from healthy hospital personnel serving on the ward when an outbreak of diarrhea among newborn occurred. These isolates were not of the same serotype as that found in the cases.

Type	ED_{50}, ml serum
011	.45
014	.0048
06	>.45

results in close agreement. Sera were stored at about –30°C; tests were performed within 3 weeks after blood collection. For absorption of serum, 14 ml of an aliquot of pool of serum was added to the washed, heat killed (56°C for 30 minutes) organisms obtained after 16 hours growth from the surface of meat extract agar in 4 Kolle flasks. The organisms were suspended in the serum, and suspension was kept at 4°C for 1 hour. Then the serum was separated from the organisms by high speed centrifugation in the cold. *Bactericidal tests.* Tests for measuring bactericidal activity of normal serum were performed by the quantitative photometric assay previously described(1). Results are expressed in terms of estimated volume (ml) of serum required to inhibit growth of 50% (ED_{50}) of test culture. *O-inagglutinability.* The O-inagglutinable status of a strain was determined by comparison of the titer of a homologous anti-O rabbit serum against organisms killed by heating for 1 hour at 100°C, and living cells. Reactions were read after 20 hours incubation in a water bath at 50°C with the killed cultures and after 2 hours at 37°C followed by 18 hours at room temperature with the living cultures. Inhibition of O-agglutination is believed to result from presence of K antigens. These are somatic antigens which act as masking antigens in a manner similar to the Vi antigens in *S. typhosa* strains (4). The K antigens are divisible into at least 3 varieties, L, A, and B based on their physical behavior.

Results. Bactericidal tests. ED_{50} of the serum pool against the isolates from normal, healthy individuals are given in Table I. The results indicated an almost 100-fold variation in susceptibility of different strains to serum bactericidal action ranging from the most resistant strains of serotype 06 and anaerogenic 011 to the most sensitive strain of serotype 014. Three of the strains tested were isolated from a healthy graduate student working with enteric bacteria (Table I,B). The serum of that individual collected about 8 months after isolation of the strains showed no greater bactericidal activity than that of other normal sera against the 3 strains. Thus, presence of non-pathogenic *E. coli* did not lead to a detectable serological response probably because the organisms were not invasive. Similar tests were performed with isolates from cases of infantile diarrhea. Results are given in Table II. Several points are of interest: relatively high level of resistance of most of these cultures, extremely high resistance of the 5 strains of serotypes 018 and 044, and marked variation in resistance of strains of serotypes 0111:B4, 0112a, 112c:B11 and 0124:B17.

O-inagglutinability. The O-inagglutinability of several *E. coli* strains and resistance of these strains to serum were compared; a significant association was observed (Table III). The highly O-inagglutinable strains such as those of serotype 02 and 0111:B4 were the most resistant to bactericidal action, although slight O-inagglutinability given by the strain of serotype 0127a, 0127b was not associated with increased resistance to normal serum bactericidal action. On the other hand, strains of serotype 07 and 086a, 086b, almost completely O-agglutinable, were extremely sensitive to normal serum bactericidal action. If the strains are ranked according to consecu-

TABLE II. Bactericidal Action of Normal Human Serum Against *E. coli* Isolates from Cases of Infantile Diarrhea.

Serotype	ED_{50}, ml serum
026:B6:H–	.31
Idem	.32 (approx.)
026:B6:H11	>.40
" : " :H32	.28
" : " :H11	.25
055:B5:H11	.22
" : " :H2	.34
" : " :H4	.30
" : " :H7	.15
" : " :H10	.29
086a, B7:H–	.19
" " :H7	.12
" " :H10	>.40
" " :H34	.23
" " :H11	>>.40
0111:B4:H–	.082
" : " :H–	.16
" : " :H2	.080
" : " :H4	.038
" : " :H6	.13
" : " :H12	.078
0112a, 112c:B11:H–	.24
Idem	.14
"	.10
"	.03
"	.11
0114:H2	.17
" :H10	.11
" : unknown	.30
0119:B14:H–	.13
" : " :H4	.11
" : " :H6	.13
" : " :H18	.23
0124:B17:H–	>>.40
" : " :H30	.13
" : " :H32	.090
" : " :H32	.092
" : " :H19	.27
0125a, 125b:B15:H21	.13
" 125c: " :H6	.12
0126:B16:H27	.10
0127:B8:H–	.058
Idem	.069
"	.092
"	.14
"	.086
0128a, 128b:B12:H8	.20
" " : " :H12	.23
" 128c: " : "	.12
018a, 18b:B20:H14	>.40
" 18c:B21:H7	>.40
044:K?:H34	>.40
" :K74:H34	>.40
" : " :H12	>.40
025:K?:H1	.13
" : " :H–	>>.40
06	>>.40
Related to 086; but not 086:B7 (K undeter.)	.29
020a, 20c:B7:H–	.19
" 20b:new K unnumbered:H26	.40

tive order in degree of O-inagglutinability and resistance to serum, a simple ordering test(6) indicates a significant degree of association ($p = <0.05$).

Specificity of normal serum bactericidal action. An aliquot of a test serum pool was absorbed with a strain of serotype 0111:B4 and another aliquot with a strain of 127a, 127b:B10. Tests were performed with the untreated serum and the 2 absorbed aliquots against 8 strains of 7 different serotypes (Table IV). Absorption of the serum pool with the 0111:B4 strain caused no reduction in its bactericidal activity against heterologous strains although activity against homologous strain was very considerably diminished. Similarly, absorption with strain 0127a, 0127b:B10 caused relatively slight reduction (less than 2-fold) in activity against heterologous strains compared to a disproportionately greater (at least 7-fold) loss against 0127 strains.

Effect of growth of cultures at different temperatures. The finding that *S. typhosa* was more susceptible to serum bactericidal action when cultivated at temperatures above or below 37°C(1) prompted an examination of this effect on *E. coli* cultures. Cultures were grown at 14°C, 37°C, 42°C and 45°C, then tested at 37°C. The results (Table V) indicate that cultures grown at 14°C and 45°C were considerably more sensitive to normal serum than those grown at 37°C, but that growth at 42°C did not result in enhanced sensitivity.

Discussion. Previous work has indicated that resistance to serum may be an important determinant of mouse virulence in *E. coli* as tested by intraperitoneal challenge(7). In this study, the data in Tables I and II did not disclose significant evidence of a relationship between resistance of a strain to serum bactericidal action and its virulence for man despite the fact that certain strains of *E. coli* isolated from normal individuals were quite sensitive to serum bactericidal action. Moreover, many *E. coli* strains isolated from normal material possessed greater resistance to normal human serum than did highly virulent strains of *S. typhosa*(1). Thus, these results indicate that resistance to normal serum cannot itself be correlated with an organism's

TABLE III. Comparison of Resistance of Strains of *E. coli* to Serum and Their O-inagglutinability.

	Strain serotype							
	02	0111:B4	0127:B8	055:B5	0128:B12	07	086a, 086b	0127a, 0127b:B10
ED$_{50}$ of normal human serum, ml	.40	.17	.16	.12	.12	.017	.014	.012
Homologous anti-O titer* with living organisms	<20	<20	40	160	40	10,240	2560	320
Homologous anti-O titer* with heat-killed organisms	5120	640	5120	10,240	640	10,240	5120	5120

* Reciprocal of anti-O agglutinin titer.

pathogenicity. Nevertheless, a relatively high level of serum resistance may represent one of the requisites for human pathogenicity since *E. coli* isolates from cases of infantile diarrhea were not among the most sensitive to serum bactericidal action.

On the other hand, an association was found between O-inagglutinability of *E. coli* strains and their resistance to normal sera (Table III). The highly O-inagglutinable strains of serotypes 02, 0111:B4 and 0127:B8 were the most resistant to bactericidal action. Slight O-inagglutinability manifested by the strain of serotype 0127a, 0127b:B10, however, provided no protection against normal serum components. This result was comparable to that observed with *S. typhosa* strains (1). Since the K antigens of *E. coli* strains are responsible for their O-inagglutinability, the results suggest that natural bactericidal antibodies are directed against O antigens of smooth strains and that antibodies against K antigen are either not present, their concentration is too low, or they are ineffective bactericidally. The increased susceptibility to serum bactericidal action of strains cultivated at temperatures other than 37°C is also probably related to loss of O-inagglutinability. A discussion of the significance of these observations in relation to the role of fever in resistance to infections with Gram-negative organisms must await studies to determine if antigenic changes *in vitro* occur in the animal host.

The absorption experiments were done to determine whether the bactericidal activities of normal serum could be attributed to one non-specific substance, to a relatively few such substances, or to many specific substances. The findings with *E. coli* (Table IV) were similar to those with other Enterobacteriaceae (1,8) and indicated a marked specificity of

TABLE IV. Bactericidal Activity of Pooled Human Serum (ED$_{50}$, ml) and Absorbed Aliquots of the Serum.

	Test strain							
Test serum	0111	127a, 127b	02	07	055	0127	0128	086a, 086b
Unabsorbed	.14	.014	>.4	.034	.19	.06	.35	.010
Abs. c̄ 127a, 127b:B10	.17	.110	"	.045	.37	.43	.60	.022
" c̄ 0111:B4	>.40	.013	"	.035	.24	.07	.40	.011

TABLE V. Resistance to Bactericidal Action of Normal Human Serum of *E. coli* Strains Cultivated at Different Temperatures.

	ED$_{50}$ of human serum (ml) against cultures grown at different temperatures			
Test culture	14°C	37°C	42°C	45°C
055, CDC #2559 - 54	.048	>.3	>.3	.17
" " #1565 - 56	.076	.13	.11	.065
0111, " #380 - "	.066	.15	.15	Not done
02, " #5086 - "	Not done	>.4	Not done	.018
0127, " #1039	"	.12	"	.0086

action. Absorption of serum with one organism resulted generally in complete inactivation against the homologous organism but little or no loss of activity against antigenically unrelated organisms.

The relationship of these findings to the nonspecific properdin system is of interest. Bactericidal activity of normal serums which were assumed to be devoid of antibody against certain bacteria has been attributed to the properdin system(9). Absorption experiments with *E. coli, S. typhosa*(1), other Gram-negative organisms(8) and also bacteriophage (10) have indicated that substances selectively removed by absorption and which may be regarded as antibodies are invariably present, however, in normal mammalian serums. Because of their low concentration, these antibodies may be unable to effect agglutination reactions, but may be detected by the bactericidal reaction, one of the most sensitive methods for antibody determination(11). In absence of antibody, normal human serum is devoid of bactericidal activity notwithstanding the presence of the components of the properdin system(8). Antibodies of marked specificity appear to play the major part, therefore, in bactericidal action of serum. Possibly, the greater susceptibility of infants to *E. coli* disease may be related to the lower concentration of these antibodies in infant's fluids, but this thesis remains to be tested.

Summary. Resistance to bactericidal action of normal human serum of *E. coli* isolates from normal children and from those with diarrhea was determined. Results indicated that serum resistance did not provide a basis for differentiation of virulent and non-virulent strains. Serum resistance was associated with the O-inagglutinability of the organisms. In addition, bactericidal action of normal human serum against *E. coli* was shown to be dependent upon natural antibodies of marked specificity.

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Desoxyribonucleic Acid (DNA)-Bentonite Flocculation Test for Lupus Erythematosus. (25620)

John Bozicevich, John P. Nasou and Donald E. Kayhoe
(Introduced by J. Freund)
(With technical assistance of Stanley B. Ward)
Laboratories of Immunology and Clinical Investigation, Nat. Inst. of Allergy and Infect. Dis., N.I.H., PHS, DHEW, Bethesda, Md.

It has been found that serum from patients with active systemic lupus erythematosus may flocculate through a wide range of titers with a suspension of a volcanic ash, bentonite, and purified desoxyribonucleic acid. Results of the test agree generally with those of lupus cell preparations. However, significant differences appear to exist especially in some patients with rheumatoid arthritis between the flocculation test and the L. E. test. In such instances, the DNA-bentonite test is negative. The present report describes the procedure for performing this test and gives some results of its clinical evaluation. More detailed clinical studies will be described later comparing the present test with lupus cell preparation and the bentonite test for rheumatoid arthritis. The test is similar to the bentonite flocculation test for rheumatoid arthritis(1) except that desoxyribonucleic acid, the coating or sensitizing agent, is used rather than gamma globulin (F II fraction). It also resembles the serological test for trichinosis described by Bozicevich *et al.*(2). Holman and Kunkel (3) demonstrated a factor (L. E. Factor) in the serum of lupus erythematosus patients which showed affinity for nucleoprotein and nuclei of cells obtained from a variety of sources. This observation is used as the basis for several tests in diagnosis of systemic lupus erythematosus. Thus, Miescher and Straessle (4) found that the "L. E. Factor" in serum agglutinated tanned red cells sensitized with desoxyribonucleic acid (DNA) or with nucleoprotein. Others applied the fluorescent antibody technic(5,6), complement fixation (7,8,9), latex agglutination(10,11), and isotope tagging procedures(12) to show this relationship.

Materials and Methods. In this test, DNA is substituted for Cohn's Fraction II of the rheumatoid arthritis test as the sensitizing agent in the flocculating system, and the surface acting agent is replaced with a solution of crystallized bovine plasma albumin as a protective colloidal substance. Bentonite, a hydrous aluminum silicate colloidal clay, is the vehicle for the flocculation reactions. Its particles are negatively charged and possess an unusually large surface to mass ratio. Bentonite powder is very stable, and is readily available. *Preparation of stock bentonite suspension.* Suspend 0.5 g of standard Volclay* (Wyoming) bentonite in 100 ml of distilled water and homogenize in a high speed blendor for 2 minutes. Allow the suspension to remain for about 5 minutes and rehomogenize. Transfer the suspension into a glass-stoppered graduate, washing the blendor with distilled water. Add sufficient distilled water to make 500 ml. Shake the graduate for one minute and allow to remain undisturbed for one hour. Place the supernatant in 6 heavy duty Pyrex centrifuge tubes of 100 ml capacity. Discard the sediment. Using an International Centrifuge, Size 2, with a No. 240 head, supernatant is centrifuged for 15 minutes at a speed of 1300 rpm (by tachometer). Relative centrifugal force is approximately 500. Discarding the sediment, supernatant is poured into 6 other 100 ml centrifuge tubes and centrifuged for 15 minutes at 1600 rpm (by tachometer). Relative centrifugal force is approximately 750. Supernatant is poured off and discarded. The accumulated sediment from the 6 tubes is resuspended in 100 ml of distilled water and homogenized for one minute. This is the stock bentonite suspension. Its adsorptive properties remain stable for several months. It is to be noted that the stock bentonite suspension will flocculate spontaneously after a few hours. When ready to use, it must be resuspended by shaking for a few seconds. *Preparation of desoxyribonucleic acid (DNA).* Place 1 mg of highly polymerized DNA† into a 20 ml test tube and add about 0.5 ml of distilled water. Sufficient time should be allowed for the DNA to swell into a translucent gel. Add 4.5 ml of distilled water and shake thoroughly. A clear, colorless, viscous solution is obtained. Prepare 24 hours prior to use and keep in refrigerator. Shake thoroughly before use. *Preparation of DNA-bentonite particles.* Place 1 ml (200 μg) of the DNA solution into a 20 ml test tube and add 10 ml of stock bentonite suspension. Shake vigorously. Add 1 ml of saturated KCl solution. Mix thoroughly and allow to remain at room temperature for one hour with occasional shaking. Add 1 ml of 1% solution in distilled water of crystallized bovine plasma albumin.‡ Invert tube several times. Avoid foaming as much as possible. Albumin solution should be allowed to remain for 10 minutes. It must be pointed out that in the rheumatoid arthritis test a surface-active agent (Tween-80) is employed to prevent the particles from clumping. Since this agent interfered with DNA-bentonite particles, a protective colloidal system is used consisting of crystallized bovine plasma albumin. Add 1 ml of 0.1% methylene blue in distilled water. Invert tube several times and centrifuge immediately for 15 minutes at 1300 rpm. Discard supernatant and add 10 ml of distilled water to the flocculated particles. Shake vigorously for one minute, then fill the tube with distilled water. Mix and centrifuge again at 1300 rpm for 15 minutes. Repeat washing once more. After the final centrifuging, discard supernatant and add 5 ml of distilled water to the residue of sensitized dyed bentonite. Shake vigorously for one minute. This is the sensitized bentonite preparation for use in the test. It must be thoroughly shaken each time prior to use. The sensitized bentonite suspension remains stable for 1 week when kept in refrigerator. It may be added that while plasma can be used for L. E. preparations, it cannot be employed for the flocculation test. The plasma often gives false positive reactions. The serum is heated at 56°C for 30 minutes. Ringed microscope slides, such as those employed in Mazzini and Kline flocculation tests (2″ × 3″) with 12 rings are used. Place 0.1 ml of undiluted serum in the first ring and then 0.1 ml of 2-fold serial dilution of serum in saline in the remaining rings. Shake the sensitized bentonite suspension and with a capillary pipette (which should deliver about 40-50 drops per ml) add one drop to each ring on the slide. Place the slide on a Boerner-type rotating machine and rotate at 110-120 times per minute for 20 minutes. Read the slide immediately with the aid of a microscope at low magnification (50×-60×). Drying of serum should be avoided. A known positive and negative serum should first be titrated each day prior to conducting the test on the routine sera, to ascertain sensitivity and specificity of the test. In reading the reaction, a 4-plus flocculation is assigned when all sensitized bentonite particles have clumped. There are 2 types—one in which several large clumps are seen and the other in which many smaller clumps are obtained. Nevertheless, practically all of the small bentonite particles are clumped. A reaction of 3-plus is recorded when approximately three-fourths of the particles are clumped. A 2-plus reaction is one in which half of the sensitized particles are clumped; a 1-plus, when one-fourth of the particles are clumped. In a negative reaction, the sensitized bentonite particles are dispersed in a colloidal suspension. The current test is more difficult to read than the bentonite flocculation test for rheumatoid arthritis, due to the adhesiveness of the DNA with the reactants in the test. Consequently, one must not select one or 2 clumps and regard the test as positive. A thorough scanning of the slide is important and the particles should be evident throughout the preparation. A reaction is regarded as positive when a 1-plus or greater flocculation occurs. With experience, the spurious aggregates can readily be distinguished from true flocculation. It must be emphasized that use of too great a quantity of DNA is to be avoided because hyaline or small fibrinous precipitation is likely to occur in presence of the albumin solution and the methylene blue dye. This makes it difficult to obtain clearcut readings.

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* American Colloid Co., Chicago, Ill.

† Worthington Biochemical Corp., Freehold, N. J.

‡ Armour Labs., Chicago, Ill.

Results. Patients with active lupus erythematosus. Sera from 8 patients have been tested. Results of the DNA-bentonite flocculation test and the L. E. preparation findings are listed in Table I. In the 8 cases there was complete agreement between flocculation test and L. E. cell preparation. The Table shows the reciprocal of the highest titer obtained by flocculation test. Serum samples tested at frequent intervals showed variation in titers.§

TABLE I. DNA-Bentonite Flocculation Tests and L.E. Cell Tests in 8 Active Cases of Systemic Lupus Erythematosus.

Age	Sex	L.E. cell	Reciprocal DNA-BFT Titer (flocculation of 1+ or greater)
31	F	Positive	512
34	F	”	256
18	F	”	64
38	F	”	32
36	F	”	32
7	F	”	32
18	F	”	16
37	F	”	8

§ To be reported.

TABLE II. DNA-Bentonite Flocculation Tests and L.E. Cell Tests in 5 Cases of Systemic Lupus Erythematosus in Remission.

Age	Sex	L.E. cell	Reciprocal DNA-BFT Titer (flocculation of 1+ or greater)
16	F	Positive	2
48	M	"	4
22	F	"	Negative
26	F	"	"
47*	F	Negative	"

* Reported as having positive L.E. cells at another hospital.

Patients in remission. Sera from 5 patients were tested (Table II). Two of these gave positive tests by both methods. Two others had positive L. E. cell preparations but gave negative flocculation tests. The remaining case gave negative results by both tests.

Patients without lupus erythematosus. One hundred and thirty-eight sera from patients without evidence of lupus erythematosus were tested. The results are shown in Table III. Patients with related and unrelated diseases were included as well as normal subjects. The DNA-bentonite flocculation test was negative in all 138 cases. The L. E. cell test was conducted on 94 of the above 138 patients, and negative results were obtained in 88 cases. To test the specificity of the flocculation test, 6 individuals, with frank rheumatoid arthritis, were selected who gave positive L. E. cell tests. Their flocculation tests were negative. Thus, it appears that the flocculation test is somewhat more specific than the L. E. cell test. However, based on the results in the remission cases of lupus erythematosus, the L. E. cell test is more sensitive than the flocculation test.

TABLE III. Results of L.E. Cell Tests with Negative DNA-Bentonite Tests on 138 N.I.H. Patients.

Diseases	No. patients	L.E. cell preparation
Rheumatoid arthritis	6	Positive
" "	5	Negative
Juvenile "	1	"
Sarcoidosis	7	"
Fever of unknown origin*	6	"
Systemic fungal disease	4	"
Endocarditis	3	"
Rheumatic heart disease	3	"
" fever, acute	3	"
Cerebral seizures	3	"
Agammaglobulinemia	3	"
Pyelonephritis, chronic	3	"
Infectious hepatitis	2	"
Intestinal parasites	2	"
Cirrhosis of liver	2	"
Empyema	2	"
Thrombocytopenic purpura	2	"
Pericarditis, etiology unknown	2	"
Well individuals	10	"
" "	44	Not tested
Misc.†	25	Negative

* Persistent fever, complete evaluation failed to reveal etiology; fever spontaneously terminated.

† Includes one case of each of following: Dermatomyositis, scleroderma, erythema multiforme, erythema nodosum, chronic glomerulonephritis, polymyositis, Sjoegren's syndrome, allergic vasculitis, chronic osteomyelitis, arteriosclerotic heart disease, asthmatic bronchitis, chronic bronchitis, cerebral palsy, Charcot-Marie-Tooth syndrome, chronic eczema, eosinophilia (persistent, etiology unknown), encephalomeningitis, lethal midline granuloma, malignant melanoma, nephrotic syndrome, osteitis deformans, pulmonary tuberculosis, Salmonella carrier, vaginal trichomoniasis, and pyoderma.

Summary. (1) The test is conducted by adding a suspension of bentonite particles previously coated with desoxyribonucleic acid (DNA) to heat inactivated serum dilutions. (2) Flocculation test showed agreement with L. E. cell test in 8 cases of lupus erythematosus. (3) L. E. cell test appeared to be more sensitive than flocculation test in cases in remission. (4) The flocculation test appeared to be more specific. In 6 selected cases of rheumatoid arthritis, the L. E. cell test was positive; on the contrary, the DNA-bentonite flocculation tests were negative.

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Relation of Mammary Tumors to Mammotropes.* I. Induction of Mammary Tumors in Rats. (25621)

UNTAE KIM AND JACOB FURTH

Children's Cancer Research Fn., Children's Medical Center, Boston, Mass., and Roswell Park Memorial Inst., Buffalo, N. Y.

It has been well established that estrogens and the pituitary gland play a major role in development of mammary gland(1) and in maintenance of growth of mammary tumors (2,3). Our studies point to a cell in the pituitary gland, named mammotrope, which produces a hormone (mammotropin) which is the direct stimulant of the mammary gland(4). Estrogens act indirectly by stimulating mammotropes(4,5). This concept is strengthened by observations described in this and the following papers(6,7), which point to the role of mammotropin in initiating as well as maintaining mammary tumors.†

Methods. Tumor induction. Mammary tumors were induced by 3-MCA in 2 highly inbred strains following Huggins' scheme(3). The rat strains used will be designated $F/_{Fu}$ and $W/_{Fu}$ following accepted terminology by mouse geneticists. The Fisher strain ($F/_{Fu}$) was obtained from Dr. Wilhelmina Dunning in 1955; the Wistar strain ($W/_{Fu}$) was a partially inbred strain purchased prior to 1945. Both were rigidly inbred and are homozygous from standpoint of transplantation. Breeding and care of rats and features of the $W/_{Fu}$ strain have been described(8). *Exp. I.* Four groups of $F/_{Fu}$ females (F-I, III, IV and V), one group of castrated $F/_{Fu}$ males (F-II), and one group of $W/_{Fu}$ females were treated with 3-MCA as indicated in Table I. Two weeks after the last treatment (112 days after start of 3-MCA feeding), a 1 mg pellet of DES was implanted s.c. in 10 rats of the F-II group. *Exp. II.* The influence of MtH on induction of mammary tumors was studied, using functional MtT as a source of this hormone(5). MtT (Strain W6) was grafted s.c. into rats of Groups W-I and W-III, 2 weeks prior to administration of 3-MCA. When a mammary tumor was detected in these rats, a biopsy was made and the tumor resected; parts of it were sectioned and parts transplanted in normal and conditioned rats, as described in the following report(6).

Results. Results of Exp. I are summarized in Tables I and II. Mortality due to toxicity of the carcinogen, indicated by severe fatty degeneration of the liver, was heavy only in Group F-V to which 3-MCA was given 6 times weekly. MT incidence was higher in 50 day old $F/_{Fu}$ rats (88 to 100%) than in those 83 days old (50%). All tumors were malignant with one exception, a fibroadenoma in a castrated male that was also given DES. One sarcoma was found in an $F/_{Fu}$ female that had multiple carcinomas at death 235 days after treatment. Most animals usually bore more than one tumor; the largest number in one rat was 13. Tumors arose in all mammary glands, perhaps most frequently in those of the thoracic region.

All $W/_{Fu}$ 3-MCA treated females developed MT within 5 months, although they received less carcinogen than $F/_{Fu}$ rats. Correspondingly, latency periods were shorter in $W/_{Fu}$ rats (Table II). All tumors in $W/_{Fu}$ rats were carcinomas.

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† *Abbreviations*: MT = mammary tumor; Mt = mammotrope; MtH = mammotropic hormone = mammotropin; MtT = mammotropic tumor; 3-MCA = 3-methylcholanthrene; DES = diethylstilbestrol.

TABLE I. Tumor Induction by Intragastric Instillation of 3-Methylcholanthrene in 10 mg doses.

Group No., sex	F-I ♀	F-III ♀	F-IV ♀	F-V ♀	F-II ♂	W-IV ♀
No. of rats:						
At start	17	22	27	23	20	18
Loss*	3	1	1	20	1	0
Rat						
Age (days)	83	51	50	70	83	56
Mean wt (g)	138	103	100	134	193	110
Treatment:						
Frequency, wkly	2×	2×	2×	6×	2×	2×
Duration, wk	14½	14½	13	6	14½	9
Total dose, mg	290	290	260	350	290	180
Tumor incidence:						
Mammary tumors:						
" adenocarcinoma	7 (50%)‡	21 (100 %)	22 (88 %)	2 (66.6%)	0	18 (100%)
" fibroadenoma	0	0	0	0	1 (20 %)	0
Squamous carcinoma:						
Periauricular	4 (50%)	14 (66.7%)	15 (62.5%)	1 (33 %)	10 (52.6%)	0
Skin	0	1 (11 %)	4 (25 %)	0	1 (33 %)	0
Mouth	1 (20%)	0	2 (10.5%)	0	1 (7.7%)	0
Leukemia	0	3 (20 %)	3 (11.5%)	0	1 (20 %)	0
Rats with tumor	9	21	25	3	10	18
Rats without tumor†	5	1	1	0	9	0

* No. dead before appearance of first tumor.
† Surviving latency period of first tumor.
‡ % calculated on basis of number of rats surviving first appearance of a particular tumor.

Incidence of squamous carcinomas of the periauricular sebaceous gland was 33 to 66% in $\mathrm{F}/_{\mathrm{Fu}}$ rats of both sexes. Other squamous carcinomas occurred in different parts of the body; 4 of these were in the mouth. None were found in $\mathrm{W}/_{\mathrm{Fu}}$ rats (Table I). Six leukemias developed in $\mathrm{F}/_{\mathrm{Fu}}$ female rats at 120 to 194 days of age, and 2 of these preceded all other tumors. No untreated controls were set up, but similar tumors were not seen at comparable ages in our breeding and ageing colony and stock of $\mathrm{F}/_{\mathrm{Fu}}$ and $\mathrm{W}/_{\mathrm{Fu}}$ rats.

Results of Exp. II are summarized in Table III. A single dose of 3-MCA produced mammary carcinomas only in animals whose mammary glands had been stimulated by MtT. Neither MtT nor 3-MCA alone was carcinogenic as here applied.

Comments. Induction of MT by intragastric instillation of 3-MCA, originated by Shay *et al.*(9) and perfected by Huggins *et al.*(3), is hereby confirmed in 2 highly inbred strains of rats. Similar tumors were not found in these strains at comparable ages, but MT and leukemia are known to occur in older animals of these strains at a much lower frequency (8). The spontaneous mammary tumors in

TABLE II. Latency Periods* of 3-MCA Induced Tumors.

Group	F-I	F-III	F-IV	F-V	F-II	W-IV
Mammary tumors	113-169 (131)	96-165 (149)	90-160 (116)	92	248†	72-154 (101)
Squamous Ca:						
Periauricular	175-200 (187)	147-255 (185)	167-263 (211)	123	140-392 (227)	
Skin		216	220-256 (235)		314	
Mouth	261		199-205		249	
Leukemia		194 (194)	120-168 (148)		249	

* Extremes with mean in parenthesis.
† Fibroadenoma.

TABLE III. Cocarcinogenesis with Mammotropes and 3-Methylcholanthrene.

Group	W-II	W-III	W-I
Treatment	3-MCA	MtT	3-MCA + MtT
No. of rats	8	8	7
MT, No. and type	1 fibroad.	1 fibroad.	6 ca., 1 fibroad.
Latency MT (days)	213	158	159-227 (170)

these and other strains of rats are usually benign. No mammary carcinomas were produced in male rats, pointing to a strong hormonal factor (*cf.* Huggins, 3) which as described in the next paper, is related to mammotropes.

Numerous squamous carcinomas and leukemias occurred in both sexes of the $F/_{Fu}$ strain, but not in the $W/_{Fu}$ strain. This may be a strain difference, possibly of genetic nature, but a confirmatory experiment is needed because total dose of carcinogen given to $W/_{Fu}$ rats was smaller and duration of treatment shorter.

An orientation experiment was performed to test our hypothesis that stimulation of the mammary gland by MtH promotes carcinogenesis. The findings strongly support this postulate. Observations of others (*cf.* Dao *et al.* 10) indicating that this hydrocarbon is concentrated in the mammary gland (as well as in fat) are in line with our hypothesis. We suppose that stimulation of the mammary gland by MtH enhances ability of the gland to trap the hydrocarbon. Male breasts also concentrate the carcinogen(10). However, they contain relatively few glandular elements but abundant fat where the carcinogen is located almost exclusively. This explains why they do not develop MT. In pregnant and lactating rats in which tumor induction rates are much higher than in virgin females(10), mammary glands are under high physiologic MtH stimulation, with intracellular as well as intraacinar concentration of lipid (milk). According to our hypothesis, male breasts stimulated by MtH and treated with 3-MCA should also develop MT (Exp. in progress).

Induction of leukemia in non-inbred W rats has already been described by Shay *et al.*(11), using 20-MCA in smaller doses over very long periods.

Summary. 1. Mammary carcinomas occurred in 50% to 100% of 64 $F/_{Fu}$ female rats surviving 90 to 96 days after initiation of intragastric instillation of 3-MCA twice weekly in 10 mg doses, and all of 18 $W/_{Fu}$ rats similarly treated, after 72 days of treatment. In castrated $F/_{Fu}$ male rats similarly treated, no mammary carcinomas developed. 2. Squamous carcinomas arising in periauricular sebaceous glands developed in about one half of both male and female $F/_{Fu}$ rats surviving 123 days, and in none of the $W/_{Fu}$ rats. Similarly, squamous carcinomas of skin and oral mucosa occurred in approximately 12% of $F/_{Fu}$ rats of both sexes and none in $W/_{Fu}$ rats. 3. Leukemias, including lymphoma (about 9%) developed in $F/_{Fu}$ rats and none in $W/_{Fu}$ rats. 4. A single dose of 3-MCA or MtH alone failed to produce mammary carcinomas in 8 $W/_{Fu}$ rats, while 6 of 7 rats receiving both, developed mammary carcinomas.

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Relation of Mammary Tumors to Mammotropes.* II. Hormone Responsiveness of 3-methylcholanthrene Induced Mammary Carcinomas.

(25622)

UNTAE KIM AND JACOB FURTH

Children's Cancer Research Fn., Children's Medical Center, Boston, Mass., and Roswell Park Memorial Inst., Buffalo, N. Y.

Mammary tumors (MT) can be readily induced in rats by oral administration of 3-methylcholanthrene (3-MCA). Many of these tumors can be inhibited by androgens, ovariectomy, or hypophysectomy and stimulated by estrogens(1). Experiments point to a cell of the pituitary, named mammotrope (Mt), the hormones of which appear to be the main stimulants of the mammary gland. Estrogens appear to exert their effect indirectly by stimulating this cell (*cf.* 2,3).

The present study was undertaken to determine the role of Mt, *i.e.*, mammotropic hormone (MtH), on growth of primary MT, induced in 33 rats with 3-MCA. All tumors were biopsied after which 13 rats were ovariectomized and 20 hypophysectomized. Each rat was studied individually and progression of tumor growth was charted. When, following ovariectomy or hypophysectomy the tumors decreased in size markedly, a functional mammotropic tumor (MtT) providing MtH, was grafted on some of the animals, while others were left untreated to serve as controls. Typical experiments are illustrated in Fig. 1-3.

Materials and methods. Tumor bearing rats of the same group(4) were matched in trios; one was hypophysectomized, one ovariectomized and one left untreated as control. Operation was performed by standard surgical procedure. Hypophysectomized animals were given Huggins' Special Diet,[†] *ad lib.* No supplementary hormones were given. Two strains of MtT were used (for supplying endogenous MtH): F4 (estrogen induced in $F/_{Fu}$ rats) and W2 (spontaneous in $W/_{Fu}$ rats). F4 grew faster than W2. MtH secretion was greater by F4, as indicated by degree of mammary gland stimulation of tumor bearing hosts. Growth of both strains was greatly stimulated by estrogens. In our terminology, such tumors are designated as functional autonomous responsive tumors. Size of MT was measured immediately before treatment and thereafter every 2 to 5 weeks. Average diameters were calculated from the 3 dimensions.

Results. In the sample experiment illustrated in Fig. 1, one rat (B) was ovariectomized when she had 8 MT, the mean diameter of which was charted. Following ovariectomy all 8 MT decreased in size. Thirty-three days following ovariectomy, MtT was grafted on this rat (to supply MtH) and this caused resumption of growth of MT. Another rat (C) had 4 MT. Hypophysectomy caused decrease in size of all 4 tumors. Upon graft of MtT all 4 tumors resumed growth, and 6 new MT appeared. The tumor of the untreated control (A) grew progressively and 2 more tumors developed on this rat at 46 days.

Fig. 2 and 3 present similar trios. Fig. 2 shows an apparently complete regression of tumor following hypophysectomy (F) but MtT caused not only reappearance of this MT, but 11 new tumors appeared on this rat (Fig. 4). In the experiment illustrated in Fig. 3 hypophysectomy was performed when the rat had 2 fairly large MT. Both decreased in size after hypophysectomy and resumed growth following an MtT graft.

The Figures also illustrate the parallelism between rate of growth of MT and MtT; *e.g.*, in Fig. 3 (H) MtT growth plateaued and so did that of MT.

Altogether, ovariectomy was performed on 13 rats bearing 50 tumors of which 36 (72%) decreased in size and 14 (28%) continued to grow with no apparent restraint (Table I). Responsive and nonresponsive tumors ap-

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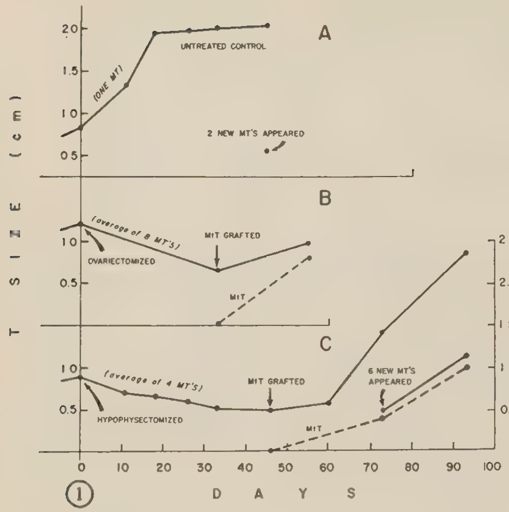
* This investigation supported by contract from Cancer Chemotherapy Nat. Service Center and grant from Nat. Cancer Inst.

[†] Obtained from Nutritional Biochemicals Corp., Cleveland, O.



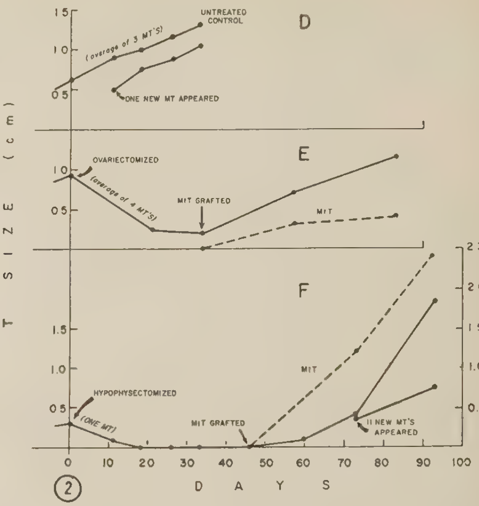
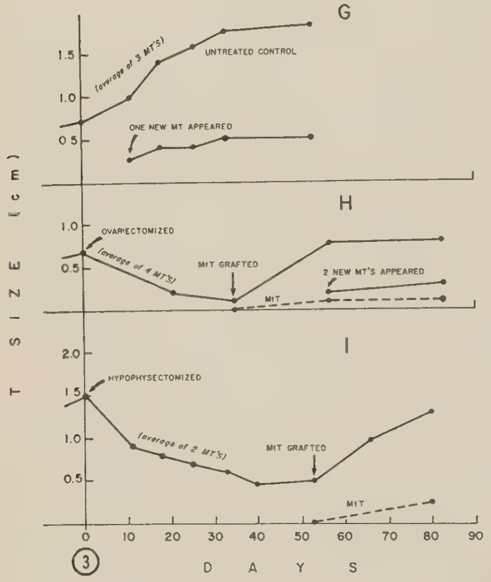


FIG. 1. Effect of ovariectomy (B) and hypophysectomy (C) on 3-MCA induced primary mammary carcinoma. Charts show decrease in tumor size following operative procedures and resumption of growth following grafts of mammotropic tumors (supplying MtH).

FIG. 2. Trio similar to that of Fig. 1. Apparent complete regression of mammary carcinoma following hypophysectomy with reappearance of this and other latent tumors following MtT graft.

FIG. 3. Trio similar to those in Fig. 1 and 2 illustrating further parallelism between MT and MtT growth rates.



peared at random in the same animal.

Hypophysectomy was performed on 20 rats bearing 59 tumors (Table II), of which 51 (86%) were inhibited. Thus, hypophysectomy was slightly more effective than ovariectomy. All tumors of all 11 rats which were inhibited by hypophysectomy resumed progressive growth following receipt of an MtT graft. In hypophysectomized rats which did not receive MtT grafts, most MT (about 54%) remained minute (measuring a few mm across) and about 37% could not be found at autopsy. About 9% of the tumors resumed progressive growth in hypophysectomized rats but at a much slower rate. No new tumors appeared in hypophysectomized rats, while this was a common event in untreated controls.

Comments. These observations are in accordance with those in man on the influence

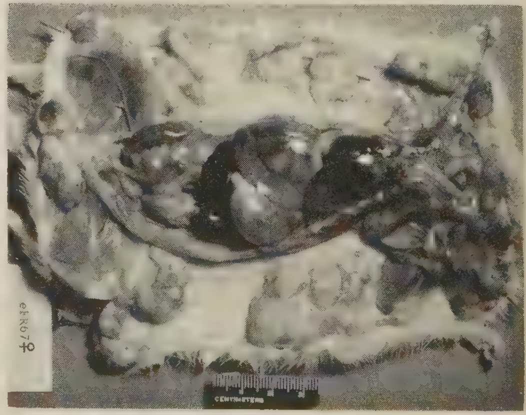


FIG. 4. Hypophysectomized rat (Fig. 2, F) with 12 macroscopic tumors appearing after MtH stimulation.

TABLE I. Effect of Ovariectomy and Mammotropic Tumor Grafts on 3-MCA Induced (Original) Tumors.

Group	No. of rats	No. of tumors	Inhibition by ovariectomy	MtT graft* No. of rats†
			%	
F-I	1	8	8 (100)	
III	5	19	11 (57)	3
IV	4	11	10 (91)	3
W-IV	4	11	17 (58)	2
Total	13	50	36 (72)	8

* Following effective ovariectomy.
† All were stimulated.

of ovariectomy and hypophysectomy on growth of mammary tumors(5,6). Rat tumors appeared more responsive to hormonal procedures than human tumors. This may be either a species characteristic or due to an earlier institution of operative procedures. Resumption of tumor growth in hypophysectomized and ovariectomized rats following grafts of functional MtT (*i.e.* MtH) is in line with our supposition that MtH secreted by Mt is the most direct stimulus of MT and estrogens act indirectly by stimulating this cell(2,3). The effectiveness of hypophysectomy in women is generally attributed to deprivation of several hormones, mainly gonadotropins, growth hormone and prolactin. Experiments in our laboratory attribute an indirect role of gonadotropin, acting through enhancement of estrogen production and link somatotropin and prolactin to the same cell, named mammotrope. Whether this cell discharges one or 2 hormones remains to be determined. Other hormones play only a minor, "permissive" non-specific function (*cf.* 2). Corticoids markedly enhance secretion but not growth of the mammary gland(2,7).

TABLE II. Effect of Hypophysectomy and Mammotropic Tumor Graft on 3-MCA Induced Tumors.

Group	No. of rats	No. of tumors	Inhibition by hypex.	MtT graft* No. of rats†
			%	
F-I	2	6	4 (67)	
III	5	9	8 (89)	4
IV	7	16	13 (81)	7
W-IV	6	28	26 (93)	
Total	20	59	51 (86)	11

* Following effective hypophysectomy.
† All were stimulated.

Summary. 1. About 72% of primary 3-MCA induced mammary carcinomas were inhibited to varying degrees by ovariectomy and 86% by hypophysectomy. 2. Most inhibited tumors remained minute; a few resumed progressive growth and a moderate number (about 37% in the hypophysectomized series) could not be identified at autopsy. 3. Grafts of functional mammotropic tumors (providing a steady supply of mammotropin) on hypophysectomized animals, whose mammary tumors have markedly decreased in size following ovariectomy or hypophysectomy, caused not only resumption of progressive tumor growth, but also appearance of numerous new mammary tumors. Thus MtH can unfold latent neoplastic potentialities.

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Relation of Mammary Tumors to Mammotropes.* III. Hormone Responsiveness of Transplanted Mammary Tumors. (25623)

UNTAE KIM, JACOB FURTH AND KELLY H. CLIFTON
Children's Cancer Research Fn., Children's Medical Center, Boston, Mass., and Roswell Park Memorial Inst., Buffalo, N. Y.

The first paper of this series(1) reported induction of mammary carcinoma (MT) in rats by 3-methylcholanthrene (3-MCA). The second paper(2) described responsiveness of primary tumors to hormones. Both papers indicate a major role of the mammotrope (Mt) in induction and growth of mammary tumors (MT) of the rat. A subcarcinogenic dose of 3-MCA caused MT only in rats whose mammary gland was stimulated by mammotropic hormone (MtH), provided by grafts of mammotropic tumors (MtT). Ovariectomy and hypophysectomy retarded many and completely arrested some primary MT. Grafts of functional MtT (*i.e.*, MtH) caused resumption of tumor growth. The purpose of this study was (a) to determine whether primary grafted MT would respond to hormonal influences in the same way as original tumors on their natural hosts, (b) to develop responsive and autonomous MT of diverse types for screening procedures and chemicals of possible therapeutic value and (c) to analyze further the role of Mt in maintaining growth of MT.†

Methods. The grafts were made either by s.c. injection of tumor mince, or by placing tumor fragments in s.c. pockets. Large tumor fragments yield tumors faster than injection of tumor mince, but the latter is a more convenient procedure when large numbers of animals are needed. In each group 6 to 10 rats, 50 to 60 days old, were used. Thirty-three induced tumors were biopsied. Parts of the tumors were sectioned and studied histologically, parts grafted on variously conditioned and normal rats of the strain of origin.

Results. Table I summarizes results of 31 transplantation experiments of 22 strains of 3-MCA induced tumors in $F/_{Fu}$ rats. All tumors were autonomous in that they grew in normal males as well as females, but, with rare exceptions, all exhibited some degree of hormonal responsiveness as indicated by slower growth rates in males than in females. Table I indicates a marked decrease in latency periods in the course of subpassages in both females and males. However, summating data obscures the marked individual differences in growth rates of tumors in males and females, the magnitude of which is a good lead on degree of estrogen responsiveness of a given mammary tumor. Some MT strains were highly estrogen responsive, others were entirely estrogen independent. Most of them were in between (Table II). Loss in degree of responsiveness and increase in growth rate are characteristic features of most, if not all MT of this series (Table I,II).

Table III compares first and second passage of 3-MCA induced MT in $W/_{Fu}$ rats. Acceptance of the graft by all 405 $W/_{Fu}$ females (Tables III, IV) indicates a practical homozygocity of this strain, while failure of 7 males to accept similar grafts indicates occasional marked hormonal (estrogen) responsiveness. This vanished, however, on the next passage.

Half of the $W/_{Fu}$ tumors were fully autonomous in the original grafts (*i.e.*, there was no difference in latency period between males and females); half were hormone responsive, as indicated either by failure to take in males or by longer latency periods and smaller size of tumors in males.

A smaller number of MT arising in irradiated $W/_{Fu}$ rats (Table IV) and of spontaneous MT of both rat strains (Table V) were also studied. While 3-MCA induced tumors were almost all carcinomas, most of those induced by radiation were fibroadenomas(3).

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* This investigation supported by contract (CCNSC) and grant from Nat. Cancer Inst.

† For tumor and host strains, procedures and key references see preceding papers(1,2). Adrenalectomized rats were maintained on 1% NaCl in drinking water. The grafted MT strains were named after the number of animal in which tumors arose.

TABLE I. Comparison of First and Subsequent Passages of Mammary Tumors in W/$_{\mathrm{Fu}}$ Rats, Induced by 3-MCA.

	Females				Males			
		No. of rats				No. of rats		
Passage	No. of exp.	Grafted	Tumor	Tumor latency*	No. of exp.	Grafted	Tumor	Tumor latency*
1st	21	193	191	(25-108) 51	11	66	64	(35-172) 97
2nd	7	137	137	(15-59) 32	8	65	63	(22-87) 52
3rd	2	66	66					
4th	1	40	40					
Total	31	436	434		19	131	127	

* days

TABLE II. Degree of Responsiveness of Grafted Tumors.

Degree of response*	1st passage No. of strains	2nd passage No. of strains
None	3	3
Slight	2	1
Moderate	2	1
Marked	3	1

* Rating was as follows: "Unresponsive" tumors were rated those which had about the same latency periods in males as in females; "slightly responsive" those tumors in which latency period in males was longer by not more than 50%; "moderately responsive" those in which this period in males was more than double; and "highly responsive" those with latency periods in males more than triple that in females.

Of 6 radiation induced tumors selected for serial transplantation, 3 were carcinomas and 3 were fibroadenomas (Table IV). All carcinoma strains of the W/$_{\mathrm{Fu}}$ rats were fully autonomous (grew equally in males and females); of the 2 fibroadenomas studied, one was fully autonomous and the other grew slightly better in females. Considered as a group, W/$_{\mathrm{Fu}}$ MT strains were less hormone responsive and had shorter latency periods than F/$_{\mathrm{Fu}}$ strains. In one W/$_{\mathrm{Fu}}$ strain latency period of the primary graft was 15 days, that of the first passage 6 days, and of the second passage 4 days. Latency period of another strain was 30 days in first passage and 18 days in second passage.

Hormonal influence. Maximal inhibition of MT in F/$_{\mathrm{Fu}}$ rats was obtained by hypophysectomy, somewhat less by ovariectomy, and almost none by adrenalectomy (Table VI). Excellent stimulation of MT growth was obtained by MtT grafts (MtH).

MtH (MtT) can even overcome the extreme inhibition in males, which is probably due to androgens (Fig. 2). In experiments shown in Fig. 1, ovariectomy and hypophysectomy were performed on groups of 6 rats when the grafted MT measured 7 to 10 mm. Six matched tumor-bearing rats were left as

TABLE IV. Summary of Transplantation Data of Mammary Tumors Occurring in X-radiated W/$_{\mathrm{Fu}}$ Rats.†

		Females		Males	
Type	Passage	No. of strains	No. rats grafted*	No. of strains	No. rats grafted*
Ca	1	3	110	2	16
	2	2	31	1	8
	3	1	8		
Fibroad.	1	3	50	2	13
Total		9	199	5	37

* All grew. † Obtained from Dr. C. J. Shellabarger.

TABLE III. Comparison of First and Second Passage of Mammary Tumors of 3-MCA Treated W/$_{\mathrm{Fu}}$ Rats.

	Females			Males			
Passage	No. of strains	No. rats grafted*	Latency, days	No. of strains	No. rats grafted	No. +	Latency, days
1	9†	206	(21-84) 40	6	47	40	(21-253) 94
2	3	54		2	18	18	

* All grew.

† 8 carcinoma and 1 fibroadenoma strains.

TABLE V. Transplantation of Spontaneous Mammary Tumors.

			Females				Males		
Rat strain	Tumor type	Passage	No. of passage	No. inj.	No. +	Latency, days	No. of passage	No. inj.*	Latency, days
F8	Fibroad.	Primary	2	38	38	13			
W1	"	"	1	10	10	60	1	15	60
		1st subp.	1	35	35	38			
W7	Ca	Primary	1	17	15	230	1	7	230
		1st subp.	1	20	20	30	1	5	30
W8	"	Primary	1	18	18	56			
		1st subp.	1	12	12	95	1	12	95

* All grew.

controls. The points in Fig. 1 represent averages. Note sharp resumption of tumor growth, following inhibition by operative procedures, upon subsequent MtT grafts.

In experiments shown in Fig. 2, grafted MT grew exceedingly slowly in 10 normal male rats. 56 days after MT graft, when the tumors were barely palpable, MtT was grafted on 5 MT bearing rats. MT growth was markedly accelerated by MtT (MtH) but remained slow without it.

In one experiment (Fig. 3), 20 rats bearing MT were divided in 2 groups when tumors measured about 7 mm; 10 rats were grafted with MtT, 10 remained as controls. Hypophysectomy was performed on all 20 rats 19 days later. MT on 10 rats bearing MtT grew progressively, while in those without MtT, shrank progressively. MtT (MtH) was necessary to maintain progressive growth of these MT in absence of the pituitary. However, the figures in Table VI are somewhat deceptive. The stimulating effect of MtH on *all* tumors listed in Table VI is due to the fact that only those tumor strains which responded to ovariectomy or hypophysectomy were challenged by MtT grafts.

TABLE VI. Hormonal Influences in Grafted Mammary Carcinomas in F/$_{\mathrm{Fu}}$ Rats.

	Inhibition				
	Ovex	Hypex	Adrex	Androg.*	Stimul. by MtH
No. of strains	8	9	4	10	7
No. of rats	47	78	18	88	46
No. of exp.	9	12	4	14	8
Effects:					
None	1	1	3	5(4)†	0
Slight	3	3	1	1(3)	0
Moderate	1	2	0	4(3)	1
Marked	4	6	0	4(4)	7

* Comparative growth of tumors in normal males vs females.

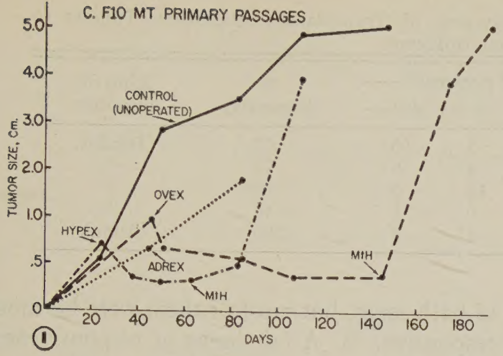
† First figure indicates prolongation of latency period, figure in parenthesis inhibition of tumor growth (size) in males *vs* females.

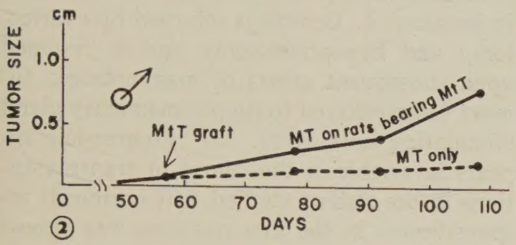
Differences in latency periods between males and females are probably due to differences in estrogen levels. Ovariectomy is more effective than would be expected on the basis of growth inhibition in males. This can be well explained by differences in estrogen levels.

There was no close relation between latency periods and hormone responsiveness; *e.g.*, F7 (Table VII), a non-responsive strain, had a latency of 27 days; 3 highly responsive strains (F5, F9, F10) had about the same latency periods (25-35 days).

Table VII attempts to answer the question whether hormone responsiveness of grafted MT's is similar to that of original tumors from which they were derived. Response to ovariectomy of original MT and first generation grafts was made in 3 cases, response to hypophysectomy in 5. There was excellent conformity between behavior of the original tumors and first generation grafts in 5 cases; in 2 there was a slight drop, and in 1 a slight gain in hormone responsiveness. On second passage, 2 tumors still remained highly responsive and 2 gained markedly in autonomy. Response to ovariectomy was compared to that of hypophysectomy in 6 first generation grafts, and in one 2nd generation graft. There was good conformity in all.

Table VIII summarizes the degree of hormonal influences on all grafted MT studied. The greatest inhibition was obtained by hypophysectomy; the least by adrenalectomy. The specificity of slight inhibition by the latter





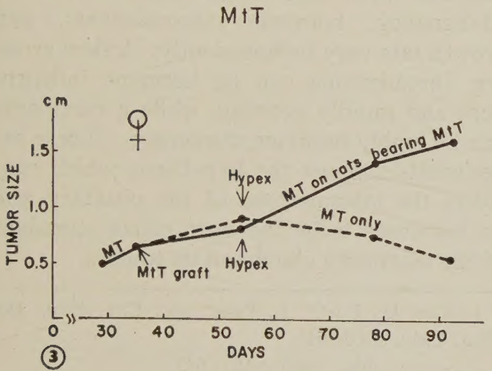


FIG. 1. Effect of ovariectomy and hypophysectomy on grafts of 3-MCA induced mammary carcinoma (Strain F10) in female rats. Each point represents avg of 6-10 rats. Note regression of tumors following surgery and resumption of growth following grafts of a MtT (supplying MtH).

FIG. 2. Exceedingly slow growth of mammary carcinoma (Strain F10) in 20 normal males (the point representing avg values) and marked stimulation of their growth in 10 of these rats following grafts of MtT.

FIG. 3. Maintenance of progressive growth of MT in hypophysectomized rats that had received MtT graft 19 days earlier and shrinkage of MT in those without it. The points are avg values on 10 rats.

TABLE VII. Comparison of Responsiveness of Original *vs* Grafted Tumor.

Strain		Response* to Ovex	Response* to Hypex
C.F1	Orig.	+	+
	Graft I	+	
F2	Orig.		+++
	Graft I	++	++
F3	Orig.		+++
	Graft I	+++	
	" II		+
F5	Orig.		+++
	Graft I		+++
	" II	+++	+++
F7	Orig.		0
	Graft I	0	0
F9	Orig.		+++
	Graft I	+++	+++
F10	Orig.		+++
	Graft I	+++	+++
	" II		+++
W1	Orig.	+++	
	Graft I	++	++
	" II		+
W4	Orig.		+++
	Graft I	+++	+++
R.W1	Orig.	++	
	Graft I	+++	+++

* + = retardation of growth, ++ = arrest of growth, +++ = regression (tumor not palpable).

(with one exception) is altogether doubtful.

Comments. Use of large numbers of rats in this transplantation series was necessitated by our aim to find stable estrogen-responsive tumor strains with varying grades of responsiveness, suitable for screening potential tumor inhibitors. This objective was not fully attained but the experiments point to technical difficulties which could be corrected. Histological examinations of mammary glands indicate that numerous cells of 3-MCA treated rats undergo neoplastic transformation throughout the glands. When grafts are made, it is likely that the more rapidly growing and independent clone outgrows the more slowly growing clone. This situation calls for clonal isolation of tumors, less intense carcinogenic treatment, and transplantation as soon as a tumor appears. There is no way known to counteract progression of a tumor, but repeated clonation may enable isolation of a desired clone. Preservation of wanted clones in the frozen state may enable their maintenance over periods of many years.

The 100% takes in numerous experiments involving 405 W/$_{\text{Fu}}$ rats of both sexes, as well as numerous other transplantation experiments, including leukemias and skin grafts (Maddock, personal communication), indicate

TABLE VIII. Comparison of Degree of Responsiveness of Transplanted Mammary Tumors to Various Hormonal Influences.

Treatment	No. of strains tested	Degree of response* 0	+	++	+++	% responsive	Kind of response
Hypex	25	2	8	5	10	92	Inhibit.
Ovex	22	5	7	4	6	78	"
Androg.*	27	8	3	10	6	70	"
Adrex	13	7	5	0	1	46	"
MtT (MtH)	10	0	0	1	8	100	Stimul.

* Retardation of tumor growth in males.

a remarkable "practical" homozygocity of the $W/_{Fu}$ strain. Transplantation data clearly indicate the autonomy of the $W/_{Fu}$ tumor strains.

Malignancy and latency periods are independent features. Fibroadenomas are benign tumors, carcinomas are malignant, yet both can grow fast or slow, both can be hormone dependent or independent, both progress in the course of passages with retention of basic features.

Experiments of Clifton *et al.* (to be published) indicate that growth of mammary glands is maintained by MtH (given in form of MtT grafts) in absence of gonads and adrenals and that cortisol markedly enhances secretion but not growth of the mammary gland. The present experiments point to a similar role of MtH with respect to growth of MT.

Summary. 1. Hormone dependence of original (spontaneous, radiation- and 3-methylcholanthrene induced) mammary tumors were compared with first and second generation grafts of the same tumor in fully histocompatible inbred strains of rats. 2. Almost all tumors, (carcinomas and fibroadenomas) were autonomous in that they grew in normal hosts of both sexes, but most of them were hormone responsive. 3. A fair index of responsiveness is the degree of preferential growth of tumors in females. 4. Growth is retarded by ovariectomy and hypophysectomy and is resumed upon subsequent grafts of mammotropic tumors (administered to supply mammary gland stimulating hormones). 5. Progression appears invariable in the course of transplantations in the strains studied, but hormonal responsiveness in the first passages was almost identical with that of primary tumors. 6. Malignancy, hormone responsiveness, and growth rate vary independently. A slow growing fibroadenoma can be hormone independent and rapidly growing, while a carcinoma can be highly hormone responsive. These experiments support the hypothesis which considers the mammotrope of the pituitary and its hormones to be the main direct stimulant of the mammary gland and its tumors.

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